Identification of Dominant Antibody-Dependent Cell-Mediated Cytotoxicity Epitopes on the Hemagglutinin Antigen of Pandemic H1N1 Influenza Virus

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Antibody-dependent cell-mediated cytotoxicity (ADCC) bridges innate and adaptive immunity, and it involves both humoral and cellular immune responses. ADCC has been found to be a main route of immune protection against viral infections *in vivo*. Hemagglutinin (HA) of influenza virus is highly immunogenic and considered the most important target for immune protection. Several potent cross-reactive HA-specific neutralizing monoclonal antibodies (MAbs) have been reported, and their conserved neutralizing epitopes have been revealed, but there has been no report so far about ADCC epitopes on HA. Here we identified two dominant ADCC epitopes, designated E1 (amino acids [aa] 92 to 117) and E2 (aa 124 to 159), on HA of pandemic H1N1 influenza virus by epitope mapping of convalescent-phase plasma IgG antibodies from six H1N1-infected human subjects in China that exhibited different levels of ADCC activity. The E1 and E2 ADCC epitopes overlapped with immunodominant epitopes of HA. Depletion of purified patient plasma IgG antibodies with EBY100 yeast cells expressing E1 or E2 decreased the ADCC activity of the IgG antibodies. E1 and E2 sequences were found to be highly conserved in H1N1 strains but less so in other subtypes of influenza A viruses. Our study may aid in designing immunogens that can elicit antibodies with high ADCC activity. Vaccine immunogens designed to include the structural determinants of potent broadly neutralizing antibodies and ADCC epitopes may confer comprehensive immune protection against influenza virus infection.

Influenza virus infection is one of the most common causes of serious respiratory illness. The outbreak of a novel H1N1 influenza virus in the year 2009 revitalized interest in understanding the epidemiological and immunological aspects of influenza virus. Virus infection induces various immune responses, of which humoral immune responses are primarily responsible for preventive and protective immunity (1, 2). Antibodies against viral antigens may provide protection either by an Fab-mediated neutralizing effect, which may interfere with the binding of virus to the cell surface receptor(s) or block other functionally important viral structures, or by Fc-mediated effector functions, including antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, and complement-dependent cytotoxicity (CDC), which destroy the infected cells. It has been reported that broadly HIV-1-neutralizing human monoclonal antibody (MAb) b12 decreased the viral load through ADCC, but not CDC, in passive immunization of rhesus macaques (3). A follow-up study of the recent RV144 phase III HIV-1 vaccine trial that demonstrated 31.2% efficacy also suggested that the high-level ADCC activity of the plasma samples of the vaccinees inversely correlated with infection risk (4, 5). ADCC against influenza virus-infected cells was first described by Greenberg et al. (6). It was well documented that the ADCC-mediated clearance of virus-infected cells occurred before infectious virus particles were released from the infected cells and before other immune responses, humoral or cellular, were initiated (7). Considering the fact that ADCC invokes a protective immune response against viral infection (8), the ADCC antibody response was incorporated as one of the important characteristics of a potential vaccine candidate by the World Health Organization (9).

A number of hemagglutinin (HA)-specific potent broadly neu-

tralizing MAbs (bnMAbs) have been reported (10–16). They target conserved epitopes either on HA2, which mediates viral fusion, or on the globular head region HA1, which interacts with the receptor. For example, HA2-specific bnMAbs CR6261 and F10 recognize conformational epitopes within the conserved A helix, while HA2-specific bnMAb 12D1 recognizes a linear epitope within the long CD helix (10, 12, 15). HA1-specific bnMAb 5J8 neutralizes a broad spectrum of 20th century H1N1 viruses and pandemic H1N1 influenza A [influenza A (H1N1)pdm] virus, and it recognizes a novel and conserved epitope between the receptor-binding pocket and the Ca2 antigenic site (13). Another HA1-specific MAb, C05, neutralizes strains from multiple subtypes of influenza A virus, and it recognizes conserved elements of the receptor-binding site by a single long heavy-chain complementarity-determining region 3 (HCDR3) loop (14). Like HIV-1-specific broadly neutralizing antibodies (bnAbs), influenza virus HA-specific bnAbs are infrequently elicited in natural infection or by vaccine immunization. Among all influenza virus HA-specific bnMAbs, CH65 may be the only MAb that was isolated from a vaccinee. CH65 also recognizes the receptor-binding pocket on HA1, mimicking in many aspects the interaction of the physiological receptor, sialic acid, with HA1 (16). Whether these HA-specific bnMAbs provide immune protection through ADCC

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FIG 1 HAI and NI titers of convalescent-phase plasma samples from seven H1N1-infected human subjects. Each plasma sample was tested for anti-HA antibodies by HAI assay. The NI titer against pandemic H1N1 A/HK/01/2009 virus was determined.

remains to be determined. There has so far been no report about an ADCC epitope(s) of the influenza virus HA antigen or its immunogenicity. In this study, we screened a panel of convalescent-phase plasma samples obtained from H1N1-infected human subjects and identified two samples with high ADCC activity against pandemic H1N1 influenza virus by using a fluorescence-based ADCC assay. We did epitope mapping of these two samples, as well as three other plasma samples with moderate to weak ADCC activity and one plasma sample with no ADCC activity, by using purified IgG antibodies and yeast display technology. We delineated potential dominant ADCC epitopes by comparing the mapping patterns against different IgG samples with different levels of ADCC activity.

MATERIALS AND METHODS

Cell lines, media, and reagents. The Raji (CCL-86) cell line was obtained from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 (Invitrogen) containing 10% heat-inactivated fetal calf serum (FCS) and 2% L-glutamine at 37°C with 5% CO₂. The following reagents were purchased: penicillin-streptomycin (Pen-Strep; Sigma), phycoerythrin (PE)-labeled anti-human IgG F(ab')₂ (Jackson ImmunoResearch), fluorescein isothiocyanate (FITC)-labeled anti-c-myc mouse IgG (Sigma), and fluorescent dyes PKH-67 (Sigma) and 7-aminoactinomycin D (7-AAD) (Invitrogen).

Convalescent-phase plasma samples from H1N1-infected human subjects. This research was authorized by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (Institutional Review Board reference number UW 11-351). All archived plasma samples used in the study were obtained from patients infected with influenza A(H1N1)pdm virus, and virus was confirmed by either reverse transcription-PCR or viral culture (17). The median time of plasma collection from the date of onset of infection was 16 months.

Purification of plasma IgG antibodies. Polyclonal IgG antibodies were purified from plasma samples by protein G affinity purification. Briefly, plasma samples were thawed, heat inactivated at 56°C for 45 min, clarified by centrifugation, and filtered through a 0.2-μm-pore-size minicapsule filter before they were loaded onto a protein G-Sepharose column (GE Biosciences) equilibrated with phosphate-buffered saline (PBS). IgG antibodies were eluted with 0.5 M acetic acid (pH 3.0), immediately neutralized with 3 M Tris (pH 9.0), and dialyzed against PBS. The purity of polyclonal IgG antibodies was confirmed by reducing and nonreducing SDS-PAGE.

HAI assay. Hemagglutination inhibition (HAI) assay was performed in V-bottom 96-well microtiter plates as previously described (18). Briefly, nonspecific inhibitors were inactivated by treating each aliquot of plasma sample with receptor-destroying enzyme (RDE; Denka Seiken, Japan) at a ratio of 1:3 (RDE/plasma) at 37°C overnight. The enzyme was heat inactivated by incubation at 56°C for 30 min. Samples were 2-fold serially diluted with a starting dilution of 1:10. An equal volume of the

FIG 2 Binding of purified IgG antibodies to H1N1-infected Raji cells by flow cytometry. All IgG samples were tested at 10 μg/ml. A nonspecific IgG sample was a secondary-antibody-only control (without a primary antibody added).

pandemic H1N1 A/HK/01/2009 virus, adjusted to approximately 4 hemagglutination units/50 μ l, was added to each well. The plates were covered and incubated at room temperature (RT) for 1 h, followed by addition of 0.5% turkey erythrocytes (RBCs) to the plasma-virus mixture and further incubation at room temperature for 30 min.

Neutralization assay. The microneutralization assay for the pandemic H1N1 A/HK/01/2009 virus was carried out in microtiter plates, with neutralization of the virus cytopathic effect in Madin-Darby canine kidney (MDCK) cells being the endpoint, as described previously (18). Briefly, plasma samples serially diluted in duplicate with a starting dilution of 1:10 were mixed with the virus at 100 50% tissue culture infective doses. Following incubation at 37°C for 2 h, the plasma-virus mixture was added to MDCK cells. At 1 h postinfection, the mixtures were removed and plasma-free minimal essential medium containing 2 μ g/ml of L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma Immunochemical) was added to each well. The plates were incubated at 37°C for 3 to 4 days, the cytopathic effect was recorded, and the highest plasma dilution that protected $\geq 50\%$ of the cells from cytopathology in the wells was determined. Positive and negative controls and virus backtitration for confirmation of the viral inoculum were included in each assay.

Preparation of infected target cells. Raji cells were used as target cells in the ADCC assay, and infected target cells were prepared as follows. Briefly, Raji lymphoblasts (19) were infected with influenza virus H1N1 A/California/04/2009 at a multiplicity to give about 80 to 95% infected cells. A sample of target cells was removed at 48 h postinfection in order to assess the percentage of infected cells on the basis of the ability of infected cells to produce hemadsorption with turkey RBCs or by flow cytometry using purified IgG antibodies from H1N1-infected patients. Note that we used H1N1 A/California/04/2009 instead of H1N1 A/HK/01/2009 to prepare infected target cells to be used in the ADCC assay. The two H1N1 strains have 99.47% homology in their HA sequences. There are three different amino acids, located at positions 100, 338, and 405. H1N1 A/California/04/2009 HA has P100, I338, and T405, while H1N1 A/HK/01/2009 HA has S100, V338, and I405.

Flow cytometry of infected target cells. Infected cells were washed twice with PBS by centrifugation at $400 \times g$ for 5 min and incubated with 5 μ g/ml purified IgG antibodies at 4°C for 2 h, followed by washing thrice with fluorescence-activated cell sorting (FACS) buffer (1% bovine serum albumin in PBS). The cells were then incubated with PE conjugated to anti-human IgG F(ab')₂ at 4°C for 1 h, followed by washing twice with FACS buffer and fixation with 2% paraformaldehyde in FACS buffer. The stained cells were analyzed on a BD flow cytometer, and the results were analyzed by FlowJo software.

Preparation of effector cells. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Paque separation of heparinized whole blood obtained from healthy volunteers and used as effector cells in the ADCC assay. Briefly, the heparinized whole blood was diluted with an equal volume of PBS containing 10% FCS and 0.5% Pen-Strep. Blood was layered over Ficoll-Paque plus (GE Healthcare) and centrifuged at $650 \times g$ for 30 min. The PBMCs were harvested and washed twice with PBS.

ADCC assay. ADCC activity was determined by a flow cytometry-based assay using two fluorescent dyes to discriminate live and dead cells (20). PKH-67, a membrane-labeling dye, was used to specifically identify the target cells. PKH-67 binds to the cell membrane, and the dye remains on the cell membrane, even after cell death, avoiding cross-contamination with effector cells. 7-AAD is excluded by viable cells but can penetrate the cell membrane of dead or dying cells and intercalate into double-stranded DNA. Briefly, PKH-67-labeled target cells and unlabeled effector cells were prepared in RPMI 1640 medium containing 10% FCS and 0.5% Pen-Strep to a cell density of 1×10^6 cells/ml and 2.5×10^7 cells/ml, respectively. Purified IgG antibodies were diluted to 5 μ g/ml and 1 μ g/ml in PBS. Fifty microliters of target cells was dispensed into a round-bottom 96-well plate in duplicate, followed by addition of 50 μ l of 5 μ g/ml or 1 μ g/ml IgG antibodies, resulting in a final concentration of 2.5 μ g/ml or

FIG 3 Percent ADCC of seven convalescent-phase plasma samples and their purified IgG antibodies. (A) Plasma samples were diluted 1:10,000 and 1:2,000; (B) purified IgG antibodies were tested at 0.5 μ g/ml and 2.5 μ g/ml. Each sample was tested in duplicate, and the average standard variation was 5%, as displayed by the error bars.

0.5 μ g/ml IgG antibodies. In the case of plasma samples used in the assay, plasma samples were diluted to a final dilution of 1:2,000 or 1:10,000. Following incubation at 37°C for 15 min, 100 μ l of effector cells was added to the target cell-IgG or plasma mixture. Effector cells (pooled PBMCs from three healthy volunteers) and target cell solutions containing no IgG and IgG antibodies from healthy volunteers were also prepared as controls. Following 2 h of incubation, 1 μ l of 7-AAD was added to the wells. Cell death was determined on a FACSaria III flow cytometer using BD FACS Diva software (BD Biosciences). A total of 5,000 target cells were acquired. Percent cell death was determined by software analysis of four identifiable cell populations: live effector cells (no dye), dead effector cells (7-AAD positive), live target cells (PKH-67 positive), and dead target cells (PKH-67 and 7-AAD double positive). Assay controls used to define cell populations included target cells alone (background cell death) and target cells with 5 μ l Triton X-100 added (maximum fluorescence). Percent ADCC was calculated as [(percent experimental lysis – percent spontaneous lysis)/(percent maximum lysis – percent spontaneous lysis)] $\times 100$, in which percent spontaneous lysis refers to the percent lysis of infected cells with effectors in the absence of plasma or IgG antibodies and percent maximum lysis refers to the percent lysis of infected cells with effectors in the presence of 1% Triton X-100. Experiments were performed in duplicate and repeated once. One representative set of data is presented in this report.

Construction of yeast-displayed HA fragment library. The gene encoding the full-length HA of influenza virus H1N1 A/HK/01/2009 was

FIG 4 Epitope mapping of six purified IgG samples. (A) The amino acid frequencies for all six IgG samples were mapped onto the HA ectodomain; (B) six IgG samples were categorized into the ADCC⁺⁺ (samples M1036 and M1037), ADCC⁺ (samples M1024, M1027, and M1039) and ADCC⁻ (sample M1089) groups, and the amino acid frequencies in each group were averaged and mapped to the H1N1 HA ectodomain. The y axes represent the frequency of each amino acid in positive clones. The x axes represent the amino acid position on H1N1 HA.

amplified by PCR using a recombinant plasmid containing the full-length HA gene as the template and a pair of primers, HAF (5'-ATGAAGGCAA TACTAGTAGTTC-3') and HAR (5'-TTAAATACATATTCTACACTG-3') (21). Two micrograms of gel-purified HA PCR products was digested with 0.9 units of DNase I (Roche) at 15°C for 15 min in a total volume of 50 µl digestion buffer (50 mM Tris-HCl, pH 7.5, 10 mM MnCl₂). The reaction was stopped by adding EDTA to a final concentration of 50 mM, followed by flash freezing in liquid nitrogen and incubation at 90°C for 10 min to inactivate the DNase I. Randomly digested PCR products were analyzed on a 2% agarose gel, and fragments ranging in size from 100 bp to 500 bp were gel extracted. The gel-purified fragments were blunt ended by using T4 DNA polymerase (New England BioLabs) and ligated to a modified pComb3X vector (the multiple-cloning sites between two SfiI sites were replaced with a SmaI restriction site) digested with SmaI. The blunt-end ligation products were electroporated into TG1 electrocompetent cells, resulting in a bacterial HA fragment library. Recombinant plasmids were prepared from the bacterial library at a large scale using a plasmid Maxi-prep kit (Qiagen), and the inserts were amplified by PCR using three sense primers, 3XYDF1 (5'-TATTTCTGTTATTGCTTCAG TTTTGCCCGAGCGGCC-3'), 3XYDF2 (5'-TATTTCTGTTATTGCTTCAGTTTTcGGCCAGGCGGCC-3'), and 3XYDF3 (5'-TATTTCT GTTATTGCTTCAGTTTTcGGCCAGGCGGCC-3'), paired with three antisense primers, 3XYDR1 (5'-ACCCTCAGAGCCACCACTAGTTGG CCGGCCTGGCC-3'), 3XYDR2 (5'-ACCCTCAGAGCCACCACTAGTT gGGCCGGCCTGGCC-3'), and 3XYDR3 (5'-ACCCTCAGAGCCACCA CTAGTTggGGCCGGCCTGGCC-3') (where the lowercase nucleotides represent insertions for correcting possible open reading frame shifts).

Each sense primer was paired with each antisense primer in the PCRs in order to avoid any clonal loss resulting from a shift in the open reading frame either in the fragment region or in the C-terminal myc tag region, or both, in the final yeast display library. All PCR products were gel purified and reamplified by PCR using high-fidelity DNA polymerase (Invitrogen) and a pair of primers, YDRDF (5'-CTTCGCTGTTTTCAATATTTTCT GTTATTGCTTCAG-3') and YDRDR (5'-GAGCCGCCACCCCTCAGA ACCGCCACCCCTCAGAGCCACCACTAG-3'), to add two overhang regions for homologous recombination with linearized yeast display plasmid pYD7, a modified vector of pCTCON2 (22). The following PCR program was used for reamplification of the inserts: an initial denaturation at 95°C for 3 min, followed by 20 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 10 min. The amplified inserts were mixed with linearized pYD7 plasmid DNA at a ratio of 3:1 (12 µg of inserts was mixed with 4 µg of linearized pYD7) and electroporated into competent yeast EBY100 cells by the lithium acetate method (23). The resultant yeast library contained 5 million individual recombinant yeast clones. Following amplification, recombinant yeast cells were aliquoted and preserved in SDCAA medium (yeast nitrogen-based Casamino Acids medium containing 20 g/liter glucose) supplemented with 15% glycerol at -80°C. Each aliquot contained 100 million yeast cells in 1 ml frozen medium.

Yeast library sorting and flow cytometry of monoclonal yeast. Induction of expression of HA fragments on the yeast cell surface was performed by following the protocol provided by Wittrup's group (22). Recombinant yeast cells were grown in SDCAA medium at 30°C for 24 h with shaking and passaged once with fresh medium to eliminate dead

FIG 5 Percent ADCC of IgG from samples M1036 and M1037 after depletion with monoclonal yeast expressing E1 or E2, or both. Each depleted IgG sample was tested at a final concentration of 2.5 μ g/ml. Undepleted IgG from samples M1036 and M1037 and IgG from samples M1036 and M1037 depleted with recombinant yeast expressing E3 were included as controls.

cells. The yeast cells were centrifuged, resuspended in SGCAA medium to an optical density at 600 nm (OD_{600}) of 0.5 to 1.0, and then induced at 20°C for 36 to 48 h with shaking. The induced yeast population was subjected to sorting against purified IgG antibodies as follows. The induced recombinant yeast population was stained with a final concentration of 500 nM purified IgG antibodies by incubation at 4°C for 3 h. Following washing twice with cold PBS, yeast cells were incubated with PE conjugated to goat anti-human IgG F(ab')₂ and FITC conjugated to mouse anti-c-myc IgG at 4°C for 1 h. Following washing thrice with cold PBS, yeast cells were sorted using a FACS Aria III flow cytometer (BD Biosciences) for populations doubly positive for PE and FITC. PE- and FITC-labeled beads and the unstained yeast library were used for calculation of compensation prior to sorting. The same gate was used for sorting of the recombinant yeast library stained with different polyclonal IgG antibodies. For each polyclonal IgG sample, 3×10^5 to 5×10^5 yeast cells were sorted. Double-positive populations were verified by flow cytometry of randomly picked monoclonal yeast cells from the population using the same primary and secondary antibodies described above.

DNA sequencing and epitope mapping. Recombinant yeast plasmids were extracted from each sorted yeast library using a yeast cell plasmid extraction kit (Omega Bio-Tek) and electroporated into *Escherichia coli* TG1 electrocompetent cells. More than 300 ampicillin-resistant yeast clones from each sorted library were sequenced using primers annealing to pYD7, and the sequences were analyzed. The recombinant clones with the inserts in the HA open reading frame and with a productive Aga2-c-myc tag were considered positive. The deduced amino acid sequences of positive clones were used for epitope mapping (<http://in.silico.ehu.es/translate/>) against the HA sequence of influenza virus H1N1 A/HK/01/2009 (GenBank accession number [ACR18920.1](#)). Insert sequences that were less than 4 aa in

length or that had an identity rate below 75% were excluded from the analysis. About 100 to 150 valid positive clones were identified for each plasma IgG sample. The frequency of each amino acid presented in the valid positive clones was counted and calibrated for the same total number of 5,000 aa for each IgG sample.

Depletion of purified IgG antibodies with recombinant yeast expressing E1 or E2. Recombinant yeast cells (10^8) surface displaying E1 or E2 were washed with PBS twice by centrifugation at $2,000 \times g$ for 5 min and incubated with 500 μ g of IgG from sample M1036 or M1037 in a total volume of 1 ml in PBS at 4°C overnight. Yeast cells were pelleted by centrifugation at $2,000 \times g$ for 5 min, the supernatant was transferred to a new preparation of 10^8 recombinant yeast cells surface displaying E1 or E2, and the mixture was incubated at RT for 1 h. Yeast cells were pelleted by centrifugation at $2,000 \times g$ for 5 min, and the supernatant was collected in new tubes. Sequential depletion with E1 and E2 was carried out by switching the recombinant yeast expressing E1 or E2 in the second round of depletion, followed by an additional round of depletion with the same recombinant yeast. Antibody concentrations in the depleted IgG samples were determined by measuring the OD_{280} using a NanoDrop apparatus.

RESULTS

Characterization of patient plasma samples. Seven convalescent-phase plasma samples were characterized for hemagglutination inhibition (HAI) and neutralization activities (Fig. 1). All plasma samples had an HAI titer of $\geq 1:160$, with plasma sample M1024 having the highest HAI titer of 1:1,280 (Fig. 1). All plasma samples showed similar neutralization endpoint dilution (neutralization inhibition [NI]) titers. Plasma samples M1017 and M1039 had an NI titer of 1:160, while all others had an NI titer of 1:320 (Fig. 1). Polyclonal antibodies were purified from these plasma samples, and the binding of purified IgG antibodies to H1N1-infected target cells was confirmed by flow cytometry (Fig. 2). All IgG samples showed similar binding activity at 10 μ g/ml, except that M1081 IgG antibodies showed relatively high binding to the infected target cells (Fig. 2). The ADCC activity of 1:10,000- and 1:2,000-diluted plasma samples was then measured by a flow cytometry-based ADCC assay. An increase in the percent cytotoxicity for infected target cells was observed in the presence of five out of seven diluted plasma samples (Fig. 3A). Three plasma samples, M1036, M1037, and M1024, exhibited high cytotoxic activity at one or both dilutions. Plasma samples M1039 and M1081 also showed cytotoxic activity that was above the average (ADCC = 10%), while the remaining two plasma samples, M1017 and M1089, did not show ADCC activity. To confirm that the IgG antibodies in the plasma samples contributed to the observed cytotoxicity, purified IgG antibodies at concentrations (0.5 μ g/ml and 2.5 μ g/ml) that were equivalent to the antibody concentrations in the diluted plasma samples were tested in the same ADCC assay (Fig. 3B). The result was largely consistent with that obtained using the diluted plasma. IgG antibodies from five out of seven samples, M1036, M1037, M1039, M1017, and M1024, had

TABLE 1 Average amino acid frequency of three immunodominant epitopes on H1N1 HA

HA epitope	Amino acid positions	Amino acid sequence	Avg frequency (%) for the following groups (samples):		
			ADCC ⁺⁺ (M1036 and M1037)	ADCC ⁺ (M1024, M1017, and M1039)	ADCC ⁻ (M1089)
E1	92–117	SWSYIVETSSDNGTCYPGDFIDYEE	51.86 $\pm$ 3.16	38.21 $\pm$ 0.72	24.11 $\pm$ 2.55
E2	124–159	SVSSFERFEIFPKISSWPNHESNKGVTAACPHAGAK	20.66 $\pm$ 2.23	32.14 $\pm$ 2.93	10.25 $\pm$ 1.19
E3	470–521	LKNNAKEIGNGCFEFYHKCDNTCMESVKNGTYD YPKYSEEAKLNREEIDGVK	15.57 $\pm$ 1.45	6.54 $\pm$ 1.17	40.17 $\pm$ 4.09

TABLE 2 Sequence conservation of E1 and E2 in H1N1 strains and all subtypes of influenza A viruses circulating in 2007, 2008, and 2009

Sequence conservation rate (%) at the indicated amino acid position (sequence) in the following HA epitope:																				
E1																				
Virus ^a	92 (S)	93 (W)	94 (S)	95 (Y)	96 (I)	97 (V)	98 (E)	99 (T)	100 (S)	101 (S)	102 (S)	103 (D)	104 (N)	105 (G)	106 (T)	107 (C)	108 (Y)	109 (P)	110 (G)	111 (D)
2007_H1N1 (1,000)	100	100	100	100	100	94	100	67	8	1	8	2	100	100	98	100	100	100	100	2
2008_H1N1 (408)	100	100	100	100	99	93	100	13	11	1	13	6	100	100	96	100	100	100	100	7
2009_H1N1 (1,000)	100	100	100	100	100	100	100	81	80	80	81	76	100	100	100	100	100	100	100	81
2007_IFA (2,325)	33	98	64	64	65	87	100	16	29	13	4	1	63	44	25	100	100	100	67	38
2008_IFA (1,339)	41	98	65	65	67	89	100	7	32	10	6	2	63	51	36	100	100	100	68	39
2009_IFA (7,880)	86	100	90	90	90	97	100	79	87	78	79	77	89	87	85	100	100	100	90	87

(Continued on next page)

above-average ADCC activity (10%) at one or both antibody concentrations, while IgG antibodies from two samples, M1081 and M1089, had below-average cytotoxicity (Fig. 3B). A discrepancy between the two sets of data was observed with sample M1017. Plasma sample M1017 had no or negative cytotoxicity, but its purified IgG antibodies had above-average cytotoxicity (Fig. 3). Based on the percent ADCC tested with the purified IgG antibodies, we categorized the IgG antibodies from six samples into three groups: strongly ADCC positive (ADCC⁺⁺; samples M1036 and M1037), ADCC positive (ADCC⁺; samples M1017, M1024, and M1039), and ADCC negative (ADCC⁻; sample M1089). IgG antibodies from all six samples were subject to epitope mapping by yeast display. Sample M1081 was excluded from the further analysis because of its below-average or negative ADCC activity in both assays.

Construction of recombinant yeast library and sorting of induced-yeast library against six IgG samples. To assess the quality of the HA fragment recombinant yeast library, we sent over 100 random yeast clones for DNA sequencing. The inserts, which had lengths ranging from 50 bp to 750 bp and an average length of 146 bp, were distributed over the full length of the HA, with no significant bias to a certain region(s), although two hot spots at amino acid positions 162 to 179 and 464 to 481 were observed (data not shown). The yeast library was induced to express HA fragments on the cell surface, and the induced yeast library was stained and incubated with individual IgG samples as the primary antibody and PE-anti-human IgG F(ab')₂ and FITC-anti-c-myc as secondary antibodies. Yeast cells doubly positive for PE and FITC were sorted and amplified. Twenty to 30 monoclonal yeast cells were randomly picked from each sorted library and tested by flow cytometry for expression of c-myc and for binding to the IgG sample used for sorting. About 50% monoclonal yeast clones were positive for binding to the IgG antibodies (the mean value was two times higher than that for the mouse IgG isotype control). All positive yeast clones were sent for DNA sequencing, and 35 to 50% of these had an insert that was in the HA open reading frame. On the basis of the small-scale sequencing result, a total of 300 to 350 monoclonal yeast cells were picked from each sorted library and sent for DNA sequencing, and about 100 to 150 valid positive clones were obtained from each sorted library.

Epitope mapping of purified IgG antibodies and identification of dominant ADCC epitopes on H1N1 HA. For each IgG sample, the amino acid sequences of all valid positive clones were

mapped to the HA sequence of influenza virus H1N1 A/HK/01/2009 (GenBank accession number [ACR18920.1](#)), and the frequency of each amino acid was counted and calibrated, so that for each IgG sample there were 5,000 aa in total. The calibrated amino acid frequencies for each IgG sample were then plotted onto the H1N1 HA sequence (Fig. 4A). We observed three immunodominant epitopes that had an average amino acid frequency of over 10 for three or more IgG samples. Two immunodominant epitopes, E1 (aa 92 to 117) and E2 (aa 124 to 159), were located in the HA1 region, and the third one, E3 (aa 470 to 521), was located in the HA2 region. To localize ADCC epitopes, the calibrated amino acid frequencies for each position within the same ADCC group were averaged, and the averaged amino acid frequencies for each ADCC group were replotted (Fig. 4B). The first two immunodominant epitopes, E1 and E2, had an average amino acid frequency that was 2-fold higher in the ADCC⁺⁺ or ADCC⁺ group than that in the ADCC⁻ group (Table 1), suggesting that E1 and E2 may be two dominant ADCC epitopes. The third immunodominant epitope, E3, showed an average amino acid frequency that was more than 2-fold higher in the ADCC⁻ group than that in the ADCC⁺⁺ or ADCC⁺ group (Table 1).

Confirmation of dominant ADCC epitopes on HA. To confirm possible ADCC epitopes, we expressed E1 and E2 on the yeast cell surface and used the recombinant yeasts to absorb two IgG samples in the ADCC⁺⁺ group, IgG samples M1036 and M1037. The IgG antibodies that absorbed with E1 or E2 alone showed significantly decreased ADCC activity compared to that for the original IgG samples and the IgG antibodies that absorbed with E3 expressed on the yeast cell surface (Fig. 5). Sequential absorption with recombinant yeast expressing E1 and E2 almost abolished the ADCC activity of the two IgG samples, confirming that E1 and E2 may be dominant ADCC epitopes on HA and antibodies specific for E1 and E2 peptides may have high ADCC activity (Fig. 5). We observed that IgG sample M1037, which absorbed with E3-expressing yeast, also showed a decrease in ADCC activity, presumably due to the removal of E3-binding antibodies that may affect ADCC activity, but the decrease in ADCC activity caused by the absorption with E1- or E2-expressing yeast was more than 2-fold more.

Conservation of E1 and E2 epitopes. We first analyzed the rate of conservation of the E1 and E2 sequences in H1N1 strains circulating from 2007 to 2009. A total of 2,408 H1N1 HA sequences downloaded from the Los Alamos Influenza Research Database

TABLE 2 (Continued)

Sequence conservation rate (%) at the indicated amino acid position (sequence) in the following HA epitope:

E1							E2														
112 (F)	113 (I)	114 (D)	115 (Y)	116 (E)	117 (E)	Avg	124 (S)	125 (V)	126 (S)	127 (S)	128 (F)	129 (E)	130 (R)	131 (F)	132 (E)	133 (I)	134 (F)	135 (P)	136 (K)	137 (T)	138 (S)
100	5	98	100	100	100	76	98	100	100	100	99	99	95	100	100	100	100	100	100	1	93
100	8	94	100	99	100	75	96	99	100	100	100	99	97	100	100	98	100	100	99	2	90
100	81	98	100	100	100	95	100	100	100	100	100	100	100	100	100	98	100	100	100	81	99
50	2	79	81	67	64	56	68	25	29	31	55	89	37	33	33	54	39	62	51	0	52
54	3	77	84	68	65	57	73	36	37	39	57	89	43	40	40	56	47	62	54	1	52
88	78	94	97	90	89	89	95	85	85	85	89	98	86	85	86	87	87	89	87	77	85

(Continued on next page)

(<http://www.fludb.org/brc/home.do?decorator=influenza>) were included in the analysis (Table 2). We found that both E1 and E2 were highly conserved in H1N1 strains from 2009 (2009_H1N1 strains), with average rates of amino acid conservation reaching 95% and 94%, respectively. E1 and E2 were relatively conserved in 2007_H1N1 and 2008_H1N1 strains, with an average rate of amino acid conservation of over 70%. Mutations mainly occurred at 6 aa positions of E1 and 11 aa positions of E2, with the rate of conservation of each amino acid being below 15% (Table 2). We then analyzed the rates of conservation of E1 and E2 in 2,325, 1,339, and 7,880 influenza A viruses isolated in 2007, 2008, and 2009, respectively. A total of 11,544 influenza A virus HA sequences downloaded from the Los Alamos Influenza Research Database were included in the analysis (Table 2). We found that both E1 and E2 were also conserved in influenza A viruses circulating in 2009, with an average conservation rate of over 80%, but much less so in influenza A viruses circulating in 2007 and 2008, with an average conservation rate of below 60% (Table 2). We found that a few residues in E1 and E2 were well conserved, with no change in any of the reported HA sequences of influenza A viruses, including E98, C107, Y108, and P109 in E1 and G148 and C153 in E2 (Table 2), being detected, suggesting their importance for HA structural integrity and/or function.

DISCUSSION

Both neutralizing and nonneutralizing antibodies may confer a cytotoxic effect that largely depends on the affinity of antibodies for Fc gamma receptors (FcγRs) (24–28). The four subclasses of human IgG differ from each other in their cytotoxic potency due to their different affinities for FcγRs. In general, the rank order for ADCC is IgG1 (+++) = IgG3 (+++) > IgG2 (±) ≥ IgG4 (±). IgG1- and IgG3-mediated ADCC relies on FcγRIIIa, which is mainly expressed on NK cells, and FcγRI, which is expressed on monocytes. Although ADCC activity is mediated by the Fc region, the Fab region of IgG, which binds to the antigen expressed on the surface of target cells, may affect Fc-mediated ADCC activity (29, 30). The mechanism for the effect of Fab on ADCC remains to be elucidated. In this study, we purified plasma antibodies by using a protein G affinity column, which purified all four IgG subclasses. In human serum, IgG1 is the most abundant subclass and accounts for 66% of total IgG antibodies, while IgG2, IgG3, and IgG4 account for 23%, 7%, and 4%, respectively. Therefore, the ADCC activity detected in this study was mainly mediated by IgG1. The similar activity of binding of purified IgG to H1N1-

infected Raji cells measured by flow cytometry suggests that the different ADCC activities of different convalescent-phase plasma IgG samples may be attributed to the different epitopes recognized by the purified IgG antibodies. Two dominant ADCC epitopes on HA were identified by differential epitope mapping of plasma IgG antibodies with different ADCC activities and confirmed by using epitope-expressing recombinant yeast-depleted IgG antibodies in the same ADCC assay. E1 is 26 aa in length, and E2 is 36 aa in length, suggesting that they may not be a single epitope but instead may be multiple epitopes and that both E1 and E2 may not be linear. Depletion of plasma IgG antibodies with E2-expressing recombinant yeast seems to be more effective than that with E1-expressing recombinant yeast (Fig. 5), suggesting that E2 may be more important than E1 in eliciting antibodies with ADCC activity. The seven humans were infected with the same H1N1 strain, but the ADCC activities of their plasma samples and purified IgG antibodies were different, and the difference did not seem to correlate with the HAI or NI titer of the plasma (Fig. 1 and 3). The mechanism for the different antibody profiles and different ADCC activities in different individuals infected with the same virus strain requires further study. The patients included in this study presented mild symptoms and recovered from influenza virus infection during the outbreak of swine flu in 2009. Their plasma had similar NI titers but different ADCC activities. It would be interesting to determine how much the ADCC activity of the plasma IgG antibodies contributed to the control of virus infection. We observed that the patient M1024 plasma sample had an HAI titer which was significantly higher than that of the other plasma samples but the purified IgG sample from patient M1024 showed moderate ADCC activity and average neutralization activity. It would also be interesting to determine if and how the HAI activity of plasma contributes to the control of virus infection. The discordance between HAI and neutralization assay results was observed in our previous study (18) and could be explained by several mechanisms. The HAI assay measures HA-specific antibody binding to HA and interfering with virus agglutination with RBCs, while the neutralization assay using live virus measures the capacity of total antibodies (not only HA-specific antibodies) for inhibiting the entry of virus into target cells. The antibodies that neutralize virus may not inhibit hemagglutination and, hence, are not detected by the HAI assay. Similarly, antibodies that inhibit hemagglutination may not have viral neutralizing activity and therefore are not detectable by the neutralization assay. In addition, the

TABLE 2 (Continued)

Sequence conservation rate (%) at the indicated amino acid position (sequence) in the following HA epitope:																					
E2																					
139 (S)	140 (W)	141 (P)	142 (N)	143 (H)	144 (D)	145 (S)	146 (N)	147 (K)	148 (G)	149 (V)	150 (T)	151 (A)	152 (A)	153 (C)	154 (P)	155 (H)	156 (A)	157 (G)	158 (A)	159 (K)	Avg
99	100	100	98	100	3	0	2	5	100	97	8	98	8	100	3	95	2	100	7	0	70
100	100	100	96	100	7	0	6	5	100	96	13	96	13	100	6	93	7	100	9	1	70
100	100	100	100	100	81	78	81	81	100	99	81	100	81	100	80	100	81	100	81	81	94
48	97	25	33	48	13	5	6	2	100	56	14	25	72	100	30	23	8	52	10	1	40
50	97	38	39	53	10	3	6	3	100	57	14	36	68	100	23	31	10	56	8	1	42
88	99	85	85	87	79	76	78	78	100	87	81	85	94	100	81	84	79	88	79	78	86

^a Values in parentheses are the number of HA sequences included in the sequence analysis.

virus strains, the host source of the red blood cells, and other nonspecific inhibitors may also affect the binding avidity in the HAI assay. Sequence conservation analysis showed that E1 and E2 were highly conserved in H1N1 strains. Although the rates of conservation of E1 and E2 were not high in other subtypes of influenza A viruses, we identified some well-conserved residues in both E1 and E2 which may be useful for designing subtype-specific vaccine immunogens. The identification of dominant ADCC epitopes on H1N1 HA shown in this study may help with the development of a universal vaccine that confers comprehensive protection against influenza virus infection.

In this study, we used H1N1-infected target cells in the ADCC assay. Both HA and neuraminidase (NA) are antigenic determinants for ADCC antibodies, but NA was found to be a minor ADCC determinant (31). The ectodomain of matrix protein 2 (M2e) of influenza A virus has been suggested to be an attractive target for a universal influenza A vaccine because the M2e sequence is highly conserved in influenza virus subtypes. Intraperitoneal or intranasal administration of M2e-based proteins/particles to mice provided 90 to 100% protection against a lethal virus challenge, and the protection was mediated by antibodies (32, 33). However, the immunogenicity of M2e alone is very weak and natural infection with influenza A viruses usually does not induce significant M2e-specific antibodies. We tested the binding of all seven convalescent-phase plasma IgG antibodies to recombinant M2e by enzyme-linked immunosorbent assay, and the titers were low overall (data not shown). We cannot exclude the possibility that M2e-specific antibodies present in IgG samples M1036 and M1037 may contribute to the killing of H1N1-infected cells, but considering the overall low titer of M2e-specific antibodies in the plasma samples and the supposedly restricted accessibility of M2e-specific antibodies to M2 on the infected cell surface in the presence of HA-specific antibodies, we assume that M2e-specific antibody-mediated ADCC activity in IgG samples M1036 and M1037 and other IgG samples may be minimal.

Various ADCC assays that differ mainly in the usage of effector cells and measurement of ADCC activity have been reported. The most popular assay was the radioactive chromium (⁵¹Cr)-release assay, which was first developed in 1968 (34). The assay was based upon the passive internalization and binding of ⁵¹Cr of sodium chromate to target cells. Lysis of the target cells by effector cells resulted in the release of the radioactive probe into the cell culture, which can be detected by a gamma counter. This assay was considered a “gold standard” for measurement of cell-mediated cyto-

toxicity. The ⁵¹Cr-release assay usually takes about 6 to 24 h to complete, depending on the type of cell, the amount of labeling, and activity measurement. This assay has a number of disadvantages, including low sensitivity, poor labeling, and high spontaneous release of isotope from some target cells. Additional problems with the ⁵¹Cr-release assay include the biohazard and disposal problems associated with the isotope. To avoid these limitations, several other methods have been developed to assess ADCC activity. These assays are based on the release of nonradioactive compounds from target cells, detection of enzymatic activity in target cells, or cell-based assays to detect dying or dead target cells by fluorometry or flow cytometry. In this study, we tested convalescent-phase human plasma and purified polyclonal IgG antibodies in a flow cytometry-based ADCC assay by differential identification of live and dead cells. We used PBMCs from healthy donors as effector cells and directly measured the dead infected cells in the presence of plasma or IgG samples. The 7-AAD dye used to discriminate live and dead or dying cells in this assay can easily pass through a dead or dying cell and intercalate with DNA, whereas the assays based on the release of nonradioactive compounds and enzymes from target cells into the culture medium require complete lysis of the target cells (20). We used NK-resistant Raji cells as the target cells in this study. Unlike MDCK cells, influenza virus-infected Raji cells do not grow fast and have low background rates of cell death in the absence of antibodies, which makes Raji an ideal cell line for the ADCC assay. In contrast, influenza virus-infected MDCK cells grow fast, and massive cell death occurs in the absence of antibodies, which gives rise to high background rates of cell death and makes it very difficult to optimize the conditions for the ADCC assay. In the present study, for some samples, we observed that more diluted plasma exhibited higher ADCC activity than less diluted plasma, and the use of IgG antibodies at a low concentration led to higher ADCC activity than the use of IgG antibodies at a high concentration (Fig. 3A and B). The same phenomenon was also observed in other studies (29, 30). It has been reported that the overall concentration of polyclonal IgG antibodies inversely affects the ADCC effect. However, it varies with the immune status of the subjects and epitope availability on the surface of the target cells. There is no conclusive study indicating the correlation of the concentration of IgG with ADCC activity. Saturation of antibodies, interference from non-ADCC antibodies present in the polyclonal antibodies, and variations in PBMCs may all contribute to this phenomenon (25, 35, 36).

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REFERENCES

- Vijaykrishna D, Smith GJ, Pybus OG, Zhu H, Bhatt S, Poon LL, Riley S, Bahl J, Ma SK, Cheung CL, Perera RA, Chen H, Shortridge KF, Webby RJ, Webster RG, Guan Y, Peiris JS. 2011. Long-term evolution and transmission dynamics of swine influenza A virus. *Nature* 473:519–522.
- Jegerlehner A, Schmitz N, Storni T, Bachmann MF. 2004. Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. *J. Immunol.* 172:5598–5605.
- Hessell AJ, Hangartner L, Hunter M, Havenith CE, Beurskens FJ, Bakker JM, Lanigan CM, Landucci G, Forthal DN, Parren PW, Marx PA, Burton DR. 2007. Fc receptor but not complement binding is important in antibody protection against HIV. *Nature* 449:101–104.
- Reks-Ngarm S, Paris RM, Chunsuttiwat S, Premisri N, Namwat C, Bowonwatanuwong C, Li SS, Kaewkungkal J, Trichavaroj R, Churikanont N, de Souza MS, Andrews C, Francis D, Adams E, Flores J, Gurunathan S, Tartaglia J, O'Connell RJ, Eamsila C, Nitayaphan S, Ngaay V, Thongcharoen P, Kunasol P, Michael NL, Robb ML, Gilbert PB, Kim JH. 2012. Extended evaluation of the virologic, immunologic, and clinical course of volunteers who acquired HIV-1 infection in a phase III vaccine trial of ALVAC-HIV and AIDSVAX B/E. *J. Infect. Dis.* 207:1195–1205.
- Reks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungkal J, Chiu J, Paris R, Premisri N, Namwat C, de Souza M, Adams E, Benenson M, Gurunathan S, Tartaglia J, McNeil JG, Francis DP, Stablein D, Bix DL, Chunsuttiwat S, Khamboonruang C, Thongcharoen P, Robb ML, Michael NL, Kunasol P, Kim JH. 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N. Engl. J. Med.* 361:2209–2220.
- Greenberg SB, Criswell BS, Six HR, Couch RB. 1977. Lymphocyte cytotoxicity to influenza virus-infected cells. II. Requirement for antibody and non-T lymphocytes. *J. Immunol.* 119:2100–2106.
- Florese RH, Demberg T, Xiao P, Kuller L, Larsen K, Summers LE, Venzon D, Cafaro A, Ensoli B, Robert-Guroff M. 2009. Contribution of nonneutralizing vaccine-elicited antibody activities to improved protective efficacy in rhesus macaques immunized with Tat/Env compared with multigenic vaccines. *J. Immunol.* 182:3718–3727.
- Gomez-Roman VR, Patterson LJ, Venzon D, Liewehr D, Aldrich K, Florese R, Robert-Guroff M. 2005. Vaccine-elicited antibodies mediate antibody-dependent cellular cytotoxicity correlated with significantly reduced acute viremia in rhesus macaques challenged with SIVmac251. *J. Immunol.* 174:2185–2189.
- World Health Organization. 1983. Immune responses to viral antigens in man and their relevance to vaccine development: memorandum from a WHO meeting. *Bull. World Health Organ.* 61:935–940.
- Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA. 2009. Antibody recognition of a highly conserved influenza virus epitope. *Science* 324:246–251.
- Ekiert DC, Friesen RH, Bhabha G, Kwaks T, Jongeneelen M, Yu W, Ophorst C, Cox F, Korse HJ, Brandenburg B, Vogels R, Brakenhoff JP, Kompier R, Koldijk MH, Cornelissen LA, Poon LL, Peiris M, Koudstaal W, Wilson IA, Goudsmit J. 2011. A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science* 333:843–850.
- Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, Wan H, Murakami A, Yammanuru A, Han T, Cox NJ, Bankston LA, Donis RO, Liddington RC, Marasco WA. 2009. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat. Struct. Mol. Biol.* 16:265–273.
- Krause JC, Tsibane T, Tumpey TM, Huffman CJ, Basler CF, Crowe JE, Jr. 2011. A broadly neutralizing human monoclonal antibody that recognizes a conserved, novel epitope on the globular head of the influenza H1N1 virus hemagglutinin. *J. Virol.* 85:10905–10908.
- Ekiert DC, Kashyap AK, Steel J, Rubrum A, Bhabha G, Khayat R, Lee JH, Dillon MA, O'Neil RE, Faynboym AM, Horowitz M, Horowitz L, Ward AB, Palese P, Webby R, Lerner RA, Bhatt RR, Wilson IA. 2012. Cross-neutralization of influenza A viruses mediated by a single antibody loop. *Nature* 489:526–532.
- Wang TT, Tan GS, Hai R, Pica N, Petersen E, Moran TM, Palese P. 2010. Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. *PLoS Pathog.* 6:e1000796. doi:10.1371/journal.ppat.1000796.
- Whittle JR, Zhang R, Khurana S, King LR, Manischewitz J, Golding H, Dormitzer PR, Haynes BF, Walter EB, Moody MA, Kepler TB, Liao HX, Harrison SC. 2011. Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. *Proc. Natl. Acad. Sci. U. S. A.* 108:14216–14221.
- Hung IF, To KK, Lee CK, Lee KL, Chan K, Yan WW, Liu R, Watt CL, Chan WM, Lai KY, Koo CK, Buckley T, Chow FL, Wong KK, Chan HS, Ching CK, Tang BS, Lau CC, Li IW, Liu SH, Chan KH, Lin CK, Yuen KY. 2011. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. *Clin. Infect. Dis.* 52:447–456.
- Chan KH, To KK, Hung IF, Zhang AJ, Chan JF, Cheng VC, Tse H, Che XY, Chen H, Yuen KY. 2011. Differences in antibody responses of individuals with natural infection and those vaccinated against pandemic H1N1 2009 influenza. *Clin. Vaccine Immunol.* 18:867–873.
- Vella S, Rocchi G, Resta S, Marcelli M, De Felici A. 1980. Antibody reactive in antibody-dependent cell-mediated cytotoxicity following influenza virus vaccination. *J. Med. Virol.* 6:203–211.
- Zaritskaya L, Shurin MR, Sayers TJ, Malyguine AM. 2010. New flow cytometric assays for monitoring cell-mediated cytotoxicity. *Expert Rev. Vaccines* 9:601–616.
- Lau SK, Chan KH, Yip CC, Ng TK, Tsang OT, Woo PC, Yuen KY. 2009. Confirmation of the first Hong Kong case of human infection by novel swine origin influenza A (H1N1) virus diagnosed using ultrarapid, real-time reverse transcriptase PCR. *J. Clin. Microbiol.* 47:2344–2346.
- Chao G, Lau WL, Hackel BJ, Szinsky SL, Lippow SM, Wittrup KD. 2006. Isolating and engineering human antibodies using yeast surface display. *Nat. Protoc.* 1:755–768.
- Gietz RD, Schiestl RH. 2007. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* 2:31–34.
- Weng WK, Levy R. 2003. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J. Clin. Oncol.* 21:3940–3947.
- Shields RL, Namenuk AK, Hong K, Meng YG, Rae J, Briggs J, Xie D, Lai J, Stadlen A, Li B, Fox JA, Presta LG. 2001. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. *J. Biol. Chem.* 276:6591–6604.
- Schlaeth M, Berger S, Derer S, Klausz K, Lohse S, Dechant M, Lazar GA, Schneider-Merck T, Peipp M, Valerius T. 2010. Fc-engineered EGF-R antibodies mediate improved antibody-dependent cellular cytotoxicity (ADCC) against KRAS-mutated tumor cells. *Cancer Sci.* 101:1080–1088.
- Nimmerjahn F, Ravetch JV. 2007. Antibodies, Fc receptors and cancer. *Curr. Opin. Immunol.* 19:239–245.
- Zhang W, Gordon M, Schultheis AM, Yang DY, Nagashima F, Azuma M, Chang HM, Borucka E, Lurie G, Sherrod AE, Iqbal S, Groshen S, Lenz HJ. 2007. FCGR2A and FCGR3A polymorphisms associated with clinical outcome of epidermal growth factor receptor expressing metastatic colorectal cancer patients treated with single-agent cetuximab. *J. Clin. Oncol.* 25:3712–3718.
- Ferrari G, Pollara J, Kozink D, Harms T, Drinker M, Freil S, Moody MA, Alam SM, Tomaras GD, Ochsenbauer C, Kappes JC, Shaw GM, Hoxie JA, Robinson JE, Haynes BF. 2011. An HIV-1 gp120 envelope human monoclonal antibody that recognizes a C1 conformational epitope mediates potent antibody-dependent cellular cytotoxicity (ADCC) activity and defines a common ADCC epitope in human HIV-1 serum. *J. Virol.* 85:7029–7036.

30. Bonsignori M, Pollara J, Moody MA, Alpert MD, Chen X, Hwang KK, Gilbert PB, Huang Y, Gurley TC, Kozink DM, Marshall DJ, Whitesides JF, Tsao CY, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Kim JH, Michael NL, Tomaras GD, Montefiori DC, Lewis GK, Devico A, Evans DT, Ferrari G, Liao HX, Haynes BF. 2012. Antibody-dependent cellular cytotoxicity-mediating antibodies from an HIV-1 vaccine efficacy trial target multiple epitopes and preferentially use the VH1 gene family. *J. Virol.* **86**:11521–11532.
31. Hashimoto G, Wright PF, Karzon DT. 1983. Antibody-dependent cell-mediated cytotoxicity against influenza virus-infected cells. *J. Infect. Dis.* **148**:785–794.
32. Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. 1999. A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nat. Med.* **5**:1157–1163.
33. El Bakkouri K, Descamps F, De Filette M, Smet A, Festjens E, Birkett A, Van Rooijen N, Verbeek S, Fiers W, Saelens X. 2011. Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. *J. Immunol.* **186**:1022–1031.
34. Brunner KT, Mauel J, Cerottini JC, Chapuis B. 1968. Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. *Immunology* **14**:181–196.
35. Umana P, Jean-Mairet J, Moudry R, Amstutz H, Bailey JE. 1999. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat. Biotechnol.* **17**:176–180.
36. Velders MP, van Rhijn CM, Oskam E, Fleuren GJ, Warnaar SO, Litvinov SV. 1998. The impact of antigen density and antibody affinity on antibody-dependent cellular cytotoxicity: relevance for immunotherapy of carcinomas. *Br. J. Cancer* **78**:478–483.