Identification of dominant ADCC epitopes on hemagglutinin antigen of pandemic H1N1 influenza virus

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ABSTRACT

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. Haemagglutinin (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 revealed, but there has been no report so far about ADCC epitopes on HA. Here we identified two dominant ADCC epitopes, designated E1 [Amino acid (AA) 92-117] and E2 (AA 124-159), on HA of pandemic H1N1 influenza virus by epitope mapping of convalescent plasma IgGs from six H1N1-infected human subjects in China that exhibited different levels of ADCC activity. E1 and E2 ADCC epitopes overlapped with immunodominant epitopes of HA. Depletion of purified patient plasma IgGs with yeast cells expressing E1 or E2 peptides decreased ADCC activity of the IgGs. 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 Abs and ADCC epitopes may confer a comprehensive immune protection against influenza virus infection.

INTRODUCTION

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 the interest to understand 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 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 mAb b12 decreased 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 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 the ADCC invokes protective immune response against viral infection (8), ADCC antibody response was incorporated as one of the important characteristics of potential vaccine candidate by World Health Organization (9).

A number of HA-specific potent broadly neutralizing mAbs (bnmAbs) have been reported (10-16). They target conserved epitopes either on HA2 that mediates viral fusion or on the globular head region HA1 that 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 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 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 remains to be determined. There has been no report so far about ADCC epitope(s) of influenza virus HA antigen and their immunogenicity. In this study we screened a panel of convalescent 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 IgGs 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 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^{o}C with 5% CO_2. The

following reagents were purchased: penicillin/streptomycin (Sigma), PE labelled anti-human IgG $(Fab')_2$ (Jackson ImmunoResearch), FITC labelled anti-c-myc mouse IgG (Sigma), and fluorescent dyes PKH-67 (Sigma) and 7-AAD (Invitrogen).

Convalescent 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 (IRB reference number: UW 11-351). All archived plasma samples used in the study were obtained from the patients infected with influenza A(H1N1)pdm virus, which were confirmed by either RT-PCR or viral culture (17). The median time of plasma collection from the date of onset of infection was 16 months.

Purification of plasma IgGs. Polyclonal IgGs 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 mini capsule filter before loaded onto a Protein G Sepharose column (GE Biosciences) equilibrated with PBS. IgGs were eluted with 0.5 M acetic acid (pH 3.0), immediately neutralized with 3M Tris (pH 9.0), and dialysed against PBS. The purity of polyclonal IgGs was confirmed by reducing and non-reducing SDS-PAGE.

Hemagglutination-inhibition (HAI) assay. HAI assay was performed in V-bottom 96-well microtiter plates as previously described (18). Briefly, non-specific 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 pandemic H1N1 A/HK/01/2009 virus, adjusted to approximately 4 HA units/50 μl was added to each well. The plates were covered and incubated at RT for 1 h followed by addition of 0.5% turkey erythrocytes to the plasma /virus mixture and further incubation at room temperature for 30 min.

Neutralization assay. The micro-neutralization assay for the pandemic H1N1 A/HK/01/2009 was carried out in microtiter plates with neutralization of the virus cytopathic effect as the endpoint in Madin-Darby canine kidney (MDCK) cells described previously (18). Briefly, serially diluted plasma samples in duplicate with a starting dilution of 1:10 were mixed with the virus with 100 50% tissue culture infective doses. Following incubation at 37°C for 2h, the plasma / virus mixture were added to MDCK cells. 1 h post infection, the mixtures were removed, and plasma-free minimal essential medium containing 2 μg/ml of TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone)-treated trypsin (TPCK-Trypsin; Sigma Immunochemical) was added to each well. The plates were incubated at 37°C for 3-4 days, and the cytopathic effect was recorded and the highest plasma dilution that protected ≥50% of the cells from cytopathology in the wells determined. Positive and negative controls and virus back titration 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 prepared as follow. Briefly, Raji lymphoblasts (19) cells were infected with influenza virus H1N1 A/California/04/2009 at a multiplicity to give about 80-95% infected cells. A sample of target cells was removed 48 h post infection in order to assess the percent infected cells based on the ability of infected cells to produce hemadsorption with turkey red blood cells (RBCs), or by flow cytometry using purified IgGs from H1N1-infected patients.

Flow cytometry of infected target cells. Infected cells were washed twice with PBS by centrifugation at 400 x g for 5 min, incubated with 5 μg/ml purified IgGs at 4°C for 2 h followed by washing thrice with FACS buffer (1% BSA 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 results 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% penicillin/streptomycin (Pen/Strep). Blood was layered over Ficoll-Paque plus (GE Healthcare) and centrifuged at 650 x 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 labelling 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-amino-actinomycin-D (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-labelled target cells and unlabelled effector cells were prepared in RPMI 1640 medium containing 10% FCS and 0.5% Pen/Strep to a cell density of 1X 10^6 cells/ml and 2.5x10^7 cells/ml, respectively. Purified IgGs were diluted to 5 µg/ml and 1 µg/ml in PBS. 50 µl of target cells were dispensed into a round-bottom 96-well plate in duplicate followed by addition of 50 µl of 5 µg/ml or 1 µg/ml IgGs, resulting in a final concentration of 2.5 µg/ml or 0.5 µg/ml IgGs. 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 µl of effector cells were added to the target cells / IgG or plasma mixture. Effector cells (pooled PBMCs from three healthy volunteers) and target cell solutions containing no IgG and IgGs from healthy volunteers were also prepared as controls. Following 2 h incubation, 1 µl of 7-AAD was added to the wells. Cell death was determined on a FACS AriaIII flow cytometer using BD FACS Diva software (BD Biosciences, USA). 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 µl Triton X-100 added (maximum fluorescence). Percent ADCC was calculated as [(% experimental lysis - % spontaneous lysis) / (% maximum lysis - % spontaneous lysis)]x100, in which "% spontaneous lysis" referred to percent lysis of infected cells with effectors in the absence of plasma or IgGs, and "% maximum lysis" referred to 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 was shown in this report.

Construction of HA fragments yeast-displayed library. The gene encoding the full-length HA of influenza virus H1N1 A/HK/01/2009 was amplified by PCR using a recombinant plasmid containing the full length HA gene as template and a pair of primers, HAF (5'-atgaaggcaatactagtagttc-3') and HAR (5'-ttaaatacatattctacactg-3') (21). 2 µg 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 $MnCl_2$). 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 DNase I. Randomly digested PCR products were analysed on 2% agarose gel and fragments ranging from 100 bp to 500 bp in size 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 Sfi I sites were replaced with a SmaI restriction site) digested with Sma I. The blunt end ligation products were electroporated into TG1 electrocompetent cells, resulting in an HA fragment bacteria library. Recombinant plasmids were large-scale prepared from the bacteria library using plasmid Maxi-prep kit (QIAGEN) and the inserts amplified by PCR using three sense primers, 3XYDF1 (5'-

tattttctgttattgcttcagttttggcccaggcggcc-3'), 3XYDF2 (5'-tattttctgttattgcttcagttttCggcccaggcggcc-3') and 3XYDF3 (5'-tattttctgttattgcttcagttttCCggcccaggcggcc-3'), paired with three antisense primers, 3XYDR1 (5'-accctcagagccaccactagttggccggcctggcc-3'), 3XYDR2 (5'-accctcagagccaccactagttgggccggcctggcc-3') and 3XYDR3 (5'-accctcagagccaccactagttGgggccggcctggcc-3'). Each sense primer was paired with each antisense primer in the PCRs in order to avoid any clonal loss resulting from open-reading frame shift 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 re-amplified by PCR using high-fidelity DNA polymerase (Invitrogen) and a pair of primers, YDRDF (5'-cttcgctgtttttcaatattttctgttattgcttcag-3') and YDRDR (5'-gagccgccaccctcagaaccgccaccctcagagccaccactag-3'), to add two overhang regions for homolog recombination with linearized yeast display plasmid pYD7, a modified vector of pCTCON2 (22). The following PCR program was used for re-amplification of the inserts: an initial denaturation at 95°C for 3 min followed by 20 cycles of (95°C, 30s, 58°C, 30s, 72°C, 30s) 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 were mixed with 4 μg of linearized pYD7), and electroporated into yeast competent cells, EBY100, by the Li-Ac 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 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 yeast cell surface was performed by following the protocol provided by Wittrup's group (22). Recombinant yeast cells were grown in SDCAA medium (yeast nitrogen-based casamino acid medium containing 20 g/l glucose) at 30°C for 24 h with

shaking and passaged once with fresh medium to eliminate dead cells. The yeast cells were centrifuged and resuspended in SGCAA medium (yeast nitrogen-based casamino acid medium containing 20g/l galactose) to an optical density at 600 nm (OD_{600}nm) of 0.5-1.0, and then induced at 20°C for 36-48 h with shaking. The induced yeast population were subject to sorting against purified IgGs as follow. The induced recombinant yeast population were stained with a final concentration of 500 nM purified IgGs 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, $(Fab')_2$ 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 FACS Aria III (BD Biosciences) for PE and FITC double positive populations. PE and FITC labelled beads and unstained yeast library were used for calculation of compensation prior to sorting. The same gate was used for sorting recombinant yeast library stained with different polyclonal IgGs. For each polyclonal IgG sample $3\text{-}5\times10^5$ yeast cells were sorted. Double positive population were verified by flow cytometry of randomly picked monoclonal yeast from the population using the same primary and secondary antibodies as described above.

DNA sequencing and epitope mapping. Recombinant yeast plasmids were extracted from each sorted yeast library using yeast cell plasmid extraction kit (Omega Bio-Tek) and electroporated into *E. 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 analysed. The recombinant clones with the inserts in the HA open-reading frame and with productive Aga2-C-myc tag were considered positive clones. The deduced amino acid (AA) sequences of positive clones were used for epitope mapping (http://insilico.ehu.es/translate/) against HA sequence of Influenza virus H1N1 A/HK/01/2009 (GenBank: ACR18920.1). The insert sequences with less than 4 AAs in length, or identity rate below 75%, were excluded from the analysis. About 100-150 valid positive clones were identified for each plasma IgG sample. The frequency of each AA

253 presented in the valid positive clones was counted and calibrated for the same total number of
254 5,000 AAs for each IgG sample.

255 **Depletion of purified IgGs with recombinant yeast expressing E1 or E2.** 10^8
256 recombinant yeast cells surface-displaying E1 or E2 were washed with PBS twice by
257 centrifugation at 2000 x g for 5 min, and incubated with 500 μg of IgGs M1036 or M1037 at
258 a total volume of 1 ml in PBS at 4°C overnight. Yeast cells were pelleted by centrifugation at
259 2000 x g for 5 min and the supernatant transferred to a new preparation of 10^8 recombinant
260 yeast cells surface-displaying E1 or E2 and incubated at RT for 1 h. Yeast cells were pelleted
261 by centrifugation at 2000 x g for 5 min and the supernatant collected in new tubes. Sequential
262 depletion with E1 and E2 was carried out by switching the recombinant yeast expressing E1
263 or E2 in the second round of depletion followed by additional round of depletion with the
264 same recombinant yeast. Antibody concentrations in the depleted IgG samples were
265 determined by measuring OD280nm using Nanodrop.

266

267 **RESULTS**

268 **Characterization of patient plasmas.** Seven convalescent plasma samples were
269 characterized for hemagglutination inhibition (HAI) and neutralization activities (Fig. 1). All
270 plasma samples had an HAI titre ≥ 1:160, with plasma M1024 having the highest HAI titre of
271 1:1,280 (Fig. 1). All plasma samples showed similar neutralization endpoint dilution (NI)
272 titers. Plasma M1017 and M1039 had an NI titer of 1:160, while all others 1:320 (Fig. 1).
273 Polyclonal antibodies were purified from these plasma samples and the binding of purified
274 IgGs to H1N1-infected target cells confirmed by flow cytometry (Fig. 2). All IgG samples
275 showed similar binding activity at 10 μg/ml except that M1081 IgGs showed relatively high
276 binding to the infected target cells (Fig. 2). ADCC activity of 1:10,000 and 1:2,000 diluted
277 plasmas was then measured by a flow cytometry-based ADCC assay. Percent increase in
278 cytotoxicity of infected target cells in the presence of five out of seven diluted plasma

samples was observed (Fig. 3A). Three plasma samples, M1036, M1037 and M1024, exhibited high cytotoxicity activity at one or both dilutions. Plasma M1039 and M1081 also showed cytotoxicity 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 IgGs in the plasma samples contributed to the observed cytotoxicity, purified IgGs at concentrations (0.5 μg/ml and 2.5 μg/ml) that were equivalent to the antibody concentrations in the diluted plasmas were tested in the same ADCC assay (Fig. 3B). The result was largely consistent with that using the diluted plasma. Five out of seven samples, IgGs M1036, M1037, M1039, M1017 and M1024, had above-average ADCC activity (10%) at one or both antibody concentrations, while two samples, IgGs M1081 and M1089 had below average cytotoxicity (Fig. 3B). Discrepancy between the two sets of data was observed with sample M1017. Plasma M1017 had no or negative cytotoxicity, but its purified IgGs had above average cytotoxicity (Fig. 3). Based on the percent ADCC tested with the purified IgGs, we categorized six IgG samples into three groups: ADCC++ (M1036, M1037), ADCC+ (M1017, M1024 and M1039), and ADCC- (M1089). All six IgG samples were subject to epitope mapping by yeast display. Sample M1081 was excluded in the further analysis for its below average or negative ADCC activity in both assays.

Construction of recombinant yeast library and sorting induced yeast library against six IgG samples. To assess the quality of HA fragments recombinant yeast library, we sent over 100 random yeast clones for DNA sequencing. The inserts with a length ranging from 50 bp to 750 bp and an average length of 146 bp were distributed over the full length HA ectodomain with no significant bias to certain region(s) although two hot spots at AA position 162-179 and 464-481 were observed (data not shown). The yeast library was induced to express HA fragments on cell surface and the induced yeast library stained were incubated with individual IgG samples as a primary antibody and PE-anti-human IgG, F(ab')2 and FITC-anti-c-myc as secondary antibodies. PE and FITC double positive yeast cells were

sorted and amplified. 20-30 monoclonal yeast 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 IgGs (mean value two times higher than mouse IgG isotype control). All positive yeast clones were sent for DNA sequencing, and 35-50 % of which had an insert that was in HA open reading frame. Based on the small scale sequencing result, a total of 300-350 monoclonal yeast were picked from each sorted library and sent for DNA sequencing, and about 100-150 valid positive clones were obtained from each sorted library.

Epitope mapping of purified IgGs and identification of dominant ADCC epitopes on H1N1 HA. For each IgG sample, the AA sequences of all valid positive clones were mapped to the HA sequence of Influenza virus H1N1 A/HK/01/2009 (GenBank: ACR18920.1) and the frequency of each AA counted and calibrated, so that for each IgG sample there were 5,000 AAs in total. The calibrated AA frequencies for each IgG sample were then plotted onto H1N1 HA sequence (Fig. 4A). We observed three immunodominant epitopes that had an average AA frequency over 10 for three or more IgG samples. Two immunodominant epitopes, HA-E1 (AA 92-117) and HA-E2 (AA 124-159), were located on HA1 region, and the third one, HA-E3 (AA 470-521), on HA2 region. To localize ADCC epitopes, the calibrated AA frequencies for each position within the same ADCC group were averaged and the averaged AA frequencies for each ADCC group re-plotted (Fig. 5B). The first two immunodominant epitopes, HA-E1 and HA-E2, had an average AA frequency that was two-fold higher in the ADCC++ or ADCC+ group than that in the ADCC- group (TABLE 1), suggesting that HA-E1 and HA-E2 may be two dominant ADCC epitopes. The third immunodominant epitope HA-E3 showed an average AA frequency that was more than two-fold higher in the ADCC- group than that in the ADCC++ or ADCC+ groups (TABLE 1).

Confirmation of dominant ADCC epitopes on HA. To comfirm possible ADCC epitopes, we expressed HA-E1 and HA-E2 on yeast cell surface and used the recombinant

yeast to absorb two IgG samples in ADCC++ group, IgGs M1036 and M1037. The IgGs absorbed with HA-E1 or HA-E2 alone showed significantly decreased ADCC activity compared to the original IgG samples and the IgGs absorbed with HA-E3 expressed on yeast cell surface (Fig. 5). Sequential absorption with recombinant yeast expressing HA-E1 and HA-E2 almost abolished the ADCC activity of the two IgG samples, confirming that HA-E1 and HA-E2 may be dominant ADCC epitopes on HA and antibodies specific for HA-E1 and HA-E2 peptides may have high ADCC activity (Fig. 5). We observed that IgG 1037 absorbed with E3-expressing yeast also showed decrease in ADCC activity, presumably due to the removal of E3-binding antibodies that may affect the 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 HA-E1 and HA-E2 epitopes. We first analyzed the conservation rate of HA-E1 and HA-E2 sequences in H1N1 strains circulating in 2007-2009. A total of 2408 H1N1 HA sequences downloaded from Los Alamos Influenza Research Database (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 2009_H1N1 strains with average AA conservation rate reached 95% and 94%, respectively. E1 and E2 were relatively conserved in 2007_ and 2008_H1N1 strains with average AA conservation rate over 70%. Mutations occurred mainly at 6 AA positions of E1 and 11 of E2 with a conservation rate of each AA below 15% (TABLE 2). We then analyzed the conservation rate of E1 and E2 in 2,325, 1,339 and 7,880 influenza A viruses (IFA) isolated in 2007, 2008 and 2009, respectively. A total of 11,544 IFA 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 IFAs circulating in 2009 with an average conservation rate over 80%, but much less so in IFAs circulating in 2007 and 2008 with an average conservation rate below 60% (TABLE 2). We found that a few residues in E1 and E2

were well conserved with no change in all reported HA sequences of IFAs, including E98, C107, Y108 and P109 in E1, and G148 and C153 in E2 (TABLE 2), suggesting their importance for HA structural integrity and / or function.

DISCUSSION

Both neutralizing and non-neutralizing antibodies may confer cytotoxicity effect largely depending on antibody affinity for Fc gamma receptors (FcrRs) (24-28). The four subclasses of human IgG differ from each other in the cytotoxic potency due to their different affinities for FcrRs. In general, the rank order is IgG1 (+++) = IgG3 (+++) > IgG2 (+/-) ≥ IgG4 (+/-) for ADCC. IgG1 and IgG3-mediated ADCC rely on FcrRIIIa that mainly expresses on NK cells and FcRI that expresses on monocytes. Although ADCC activity is mediated by the Fc region, the Fab region of IgG that binds to the antigen expressed on the surface of target cells may affect Fc-mediated ADCC activity (29, 30). The mechanism for Fab effect on ADCC remains to be elucidated. In this study, we purified plasma antibodies by using Protein G affinity column, which purified all four IgG subclasses. In human sera, IgG1 is the most abundant subclass and accounts for 66% of total IgGs, while IgG2, IgG3, IgG4 account for 23%, 7% and 4%, respectively. Therefore, the ADCC activity detected in this study was mediated mainly by IgG1s. Similar binding activity of purified IgGs to H1N1-infected Raji cells as measured by flow cytometry suggests that different ADCC activities of different convalescent plasma IgG samples may be attributed to different epitopes recognized by the purified IgGs. Two dominant ADCC epitopes on HA were identified by differential epitope mapping of plasma IgGs with different ADCC activities, and confirmed by using epitope-expressing recombinant yeast depleted IgGs in the same ADCC assay. E1 has 26 AAs and E2 36 AAs in length, suggesting that they may not be a single epitope, but multiple epitopes instead, and that both E1 and E2 may not be linear epitopes. Depletion of plasma IgGs with E2-expressing recombinant yeast seems more effective than that with E1-expressing

383 recombinant yeast (Fig. 5), suggesting that E2 may be more important than E1 in eliciting
384 antibodies with ADCC activity. The seven human individuals were infected with same H1N1
385 strain, but the ADCC activity of their plasmas and purified IgGs were different, and the
386 difference did not seem to correlate with the HAI and NI titers of the plasma (Fig. 1 and 3).
387 The mechanism for the different antibody profiles and different ADCC activities in different
388 individuals infected with the same virus strain requires further study. The patients included in
389 this study presented mild symptoms and recovered from influenza virus infection during the
390 outbreak of swine flu in 2009. Their plasma had a similar NI titre, but different ADCC
391 activity. It would be interesting to determine how much the ADCC activity of the plasma
392 IgGs contribute to the control of virus infection. We observed that patient M1024 plasma had
393 an HAI titer which was significantly higher than that of other plasmas, but purified IgG
394 M1024 showed moderate ADCC activity and an average neutralization activity. It would be
395 also interesting to determine if and how HAI activity of the plasma contributes to the control
396 of virus infection. The discordance between HAI and neutralization assay results has been
397 observed in our previous study (18), and could be explained by several mechanisms. The HAI
398 assay measures HA-specific antibodies in binding to HA and interfering with virus
399 agglutination with RBC, while the neutralization assay using live virus measures the capacity
400 of total antibodies (not only HA-specific antibodies) for inhibiting the entry of virus to target
401 cells. The antibodies that neutralize virus may not inhibit hemagglutination and hence are not
402 detected by the HAI assay. Similarly, antibodies that inhibit hemagglutination may not have
403 viral neutralizing activity and therefore are not detectable by the neutralization assay. In
404 addition, virus strains, host source of the red blood cells and other non-specific inhibitors may
405 also affect the binding avidity in HAI assay. Sequence conservation analysis showed that E1
406 and E2 were highly conserved in H1N1 strains. Although the conservation rates of E1 and E2
407 were not high in other subtypes of influenza A viruses, we identified some well conserved
408 residues in both E1 and E2, which may be useful for designing subtype-specific vaccine

409 immunogens. The identification of dominant ADCC epitopes on H1N1 HA shown in this
410 study may help develop universal vaccine that confers comprehensive protection against
411 influenza virus infection.

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

428 Various ADCC assays have been reported that differ mainly in the usage of effector
429 cells and measurement of ADCC activity. The most popular assay was the radioactive
430 chromium (^{51}Cr)-release assay, which was first developed in 1968 (34). The assay was based
431 upon the passive internalization and binding of ^{51}Cr of sodium chromate to target cells. Lysis
432 of the target cells by effector cells resulted in the release of the radioactive probe into the cell
433 culture, which can be detected by a γ-counter. This assay was considered a 'gold standard' to
434 measure cell-mediated cytotoxicity. The ^{51}Cr release assay usually takes about 6 to 24h to

complete depending on the type of cells, amount of labelling and activity measurement. This assay has a number of disadvantages, including low sensitivity, poor labelling and high spontaneous release of isotope from some target cells. Additional problems with the ^{51}Cr-release assay include biohazard and disposal problems 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, or 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 human plasma and purified polyclonal IgGs 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 plasmas or IgGs. The 7AAD dye used in this assay to discriminate live and dead or dying cells 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 to culture medium require complete lysis of the target cells (20). We used NK-resistant Raji cells as target cells in this study. Unlike MDCK cells, influenza virus infected Raji cells do not grow fast and have low background 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 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 IgGs at a low concentration led to higher ADCC activity than the IgGs at a high concentration (Fig. 3A, 3B). The same phenomenon was also observed in other studies (29, 30). It has been reported that the overall concentration of polyclonal IgGs affect inversely the ADCC effect. However, it varies with immune status of the subjects and epitope availability on the surface of the target cells. There is no conclusive study indicating the

correlation of concentration of IgG with ADCC activity. Saturation of antibodies, interference of non-ADCC antibodies present in the polyclonal antibodies, and variation of PBMCs may all contribute to this phenomenon (25, 35, 36).

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FIGURE LEGEND

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

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

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

FIG 4 Epitope mapping of six purified IgG samples. A: The AA frequencies for all six IgG samples were mapped onto HA ectodomain. B: Six IgG samples were grouped into ADCC++ (M1036 and M1037), ADCC+ (M1024, M1027 and M1039) and ADCC- (M1089) samples, and the AA frequencies averaged in each group and mapped to H1N1 HA ectodomain. Y axis represents the frequency of each AA in positive clones. X axis represents AA position on H1N1 HA.

FIG 5 Percent ADCC of IgGs 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 μg/ml. Undepleted IgGs M1036 and M1037, and IgGs M1036 and 1037 depleted with recombinant yeast expressing E3 were included as controls.

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FIG 1

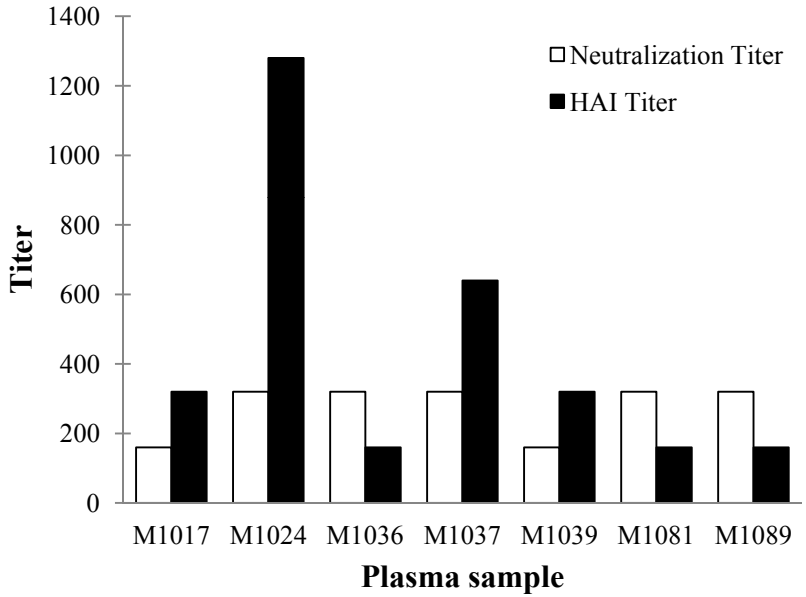
1400
1200
1000
800
600
400
200
0
Titer
Neutralization Titer
HAI Titer
M1017
M1024
M1036
M1037
M1039
M1081
M1089
Plasma sample

FIG 2

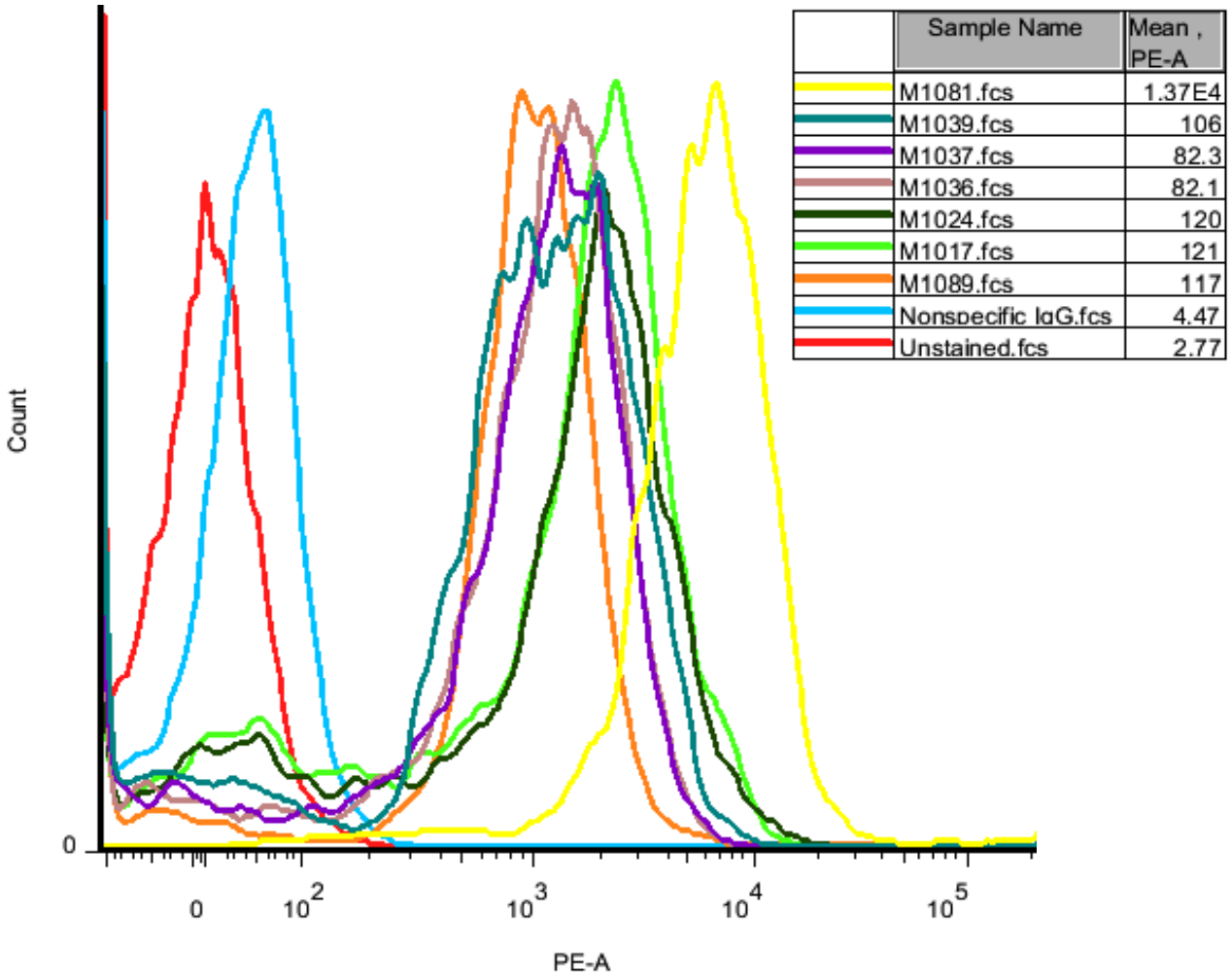
Sample Name
Mean , PE-A
M1081.fcs 1.37E4
M1039.fcs 106
M1037.fcs 82.3
M1036.fcs 82.1
M1024.fcs 120
M1017.fcs 121
M1089.fcs 117
Nonspecific IgG.fcs 4.47
Unstained.fcs 2.77
Count
0
0
10^2
10^3
10^4
10^5
PE-A

FIG 3

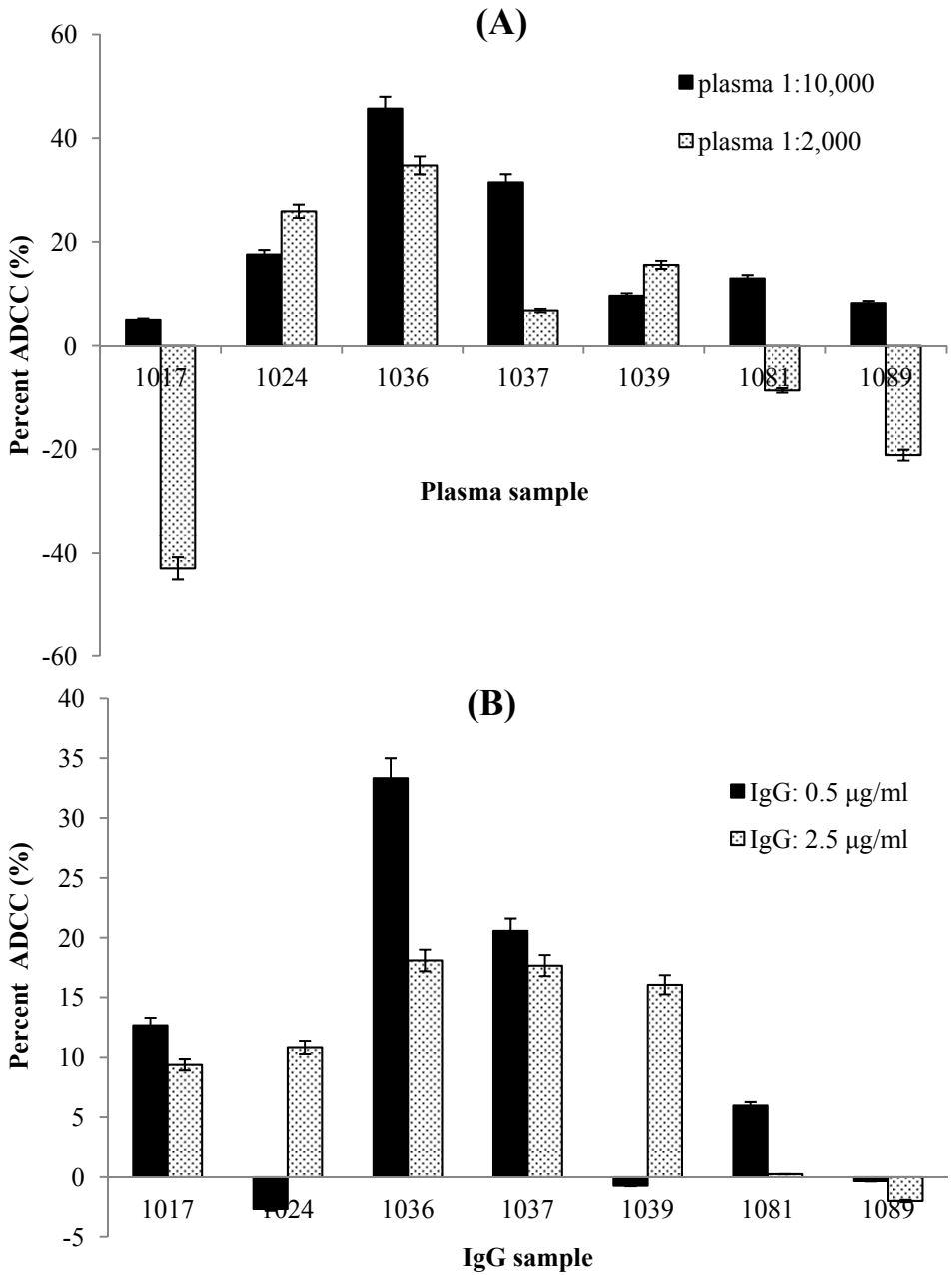
(A)
plasma 1:10,000
plasma 1:2,000
Percent ADCC (%)
60
40
20
0
-20
-40
-60
1017
1024
1036
1037
1039
1081
1089
Plasma sample
(B)
IgG: 0.5 µg/ml
IgG: 2.5 µg/ml
Percent ADCC (%)
40
35
30
25
20
15
10
5
0
-5
1017
1024
1036
1037
1039
1081
1089
IgG sample

FIG 4

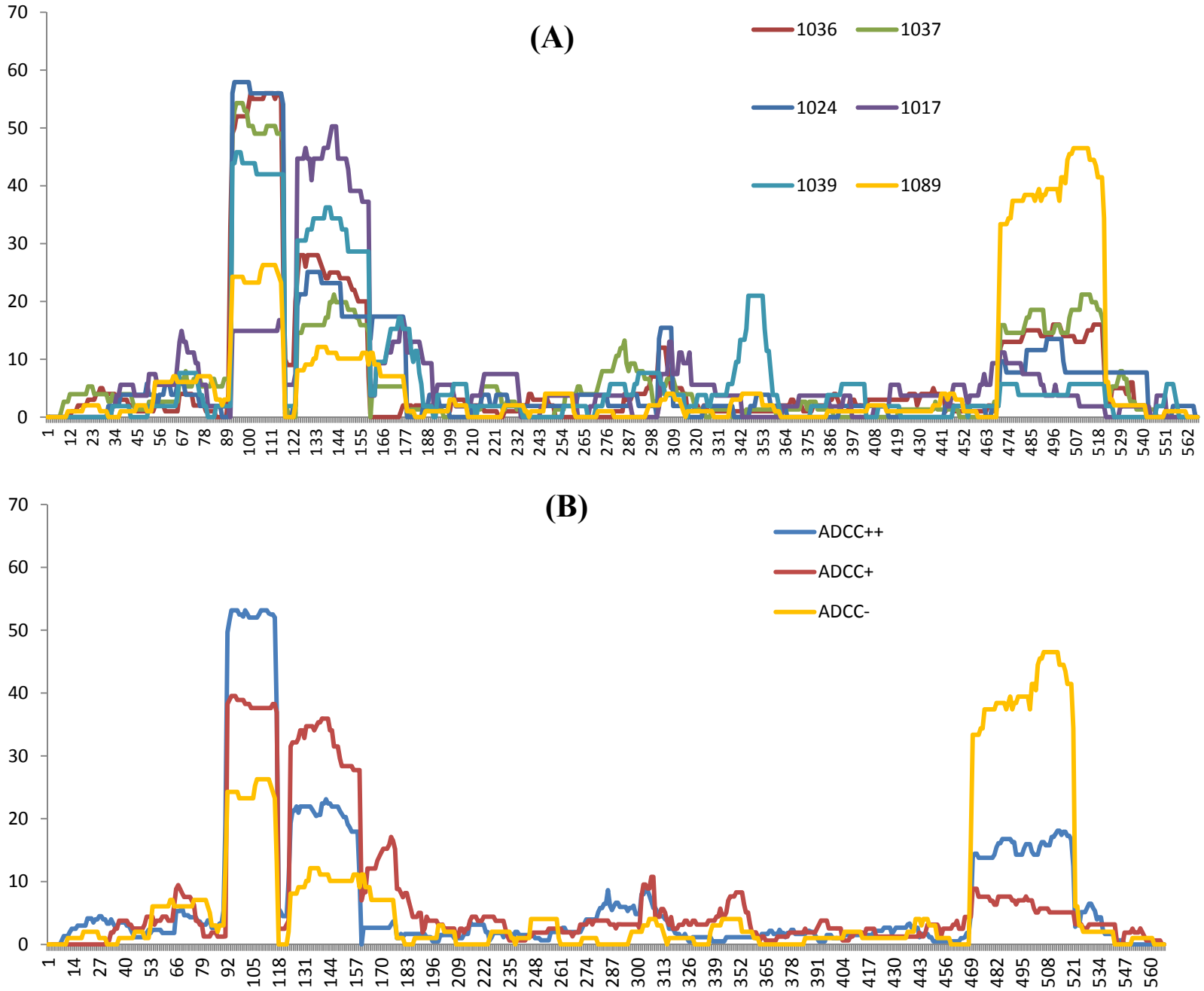

FIG 5

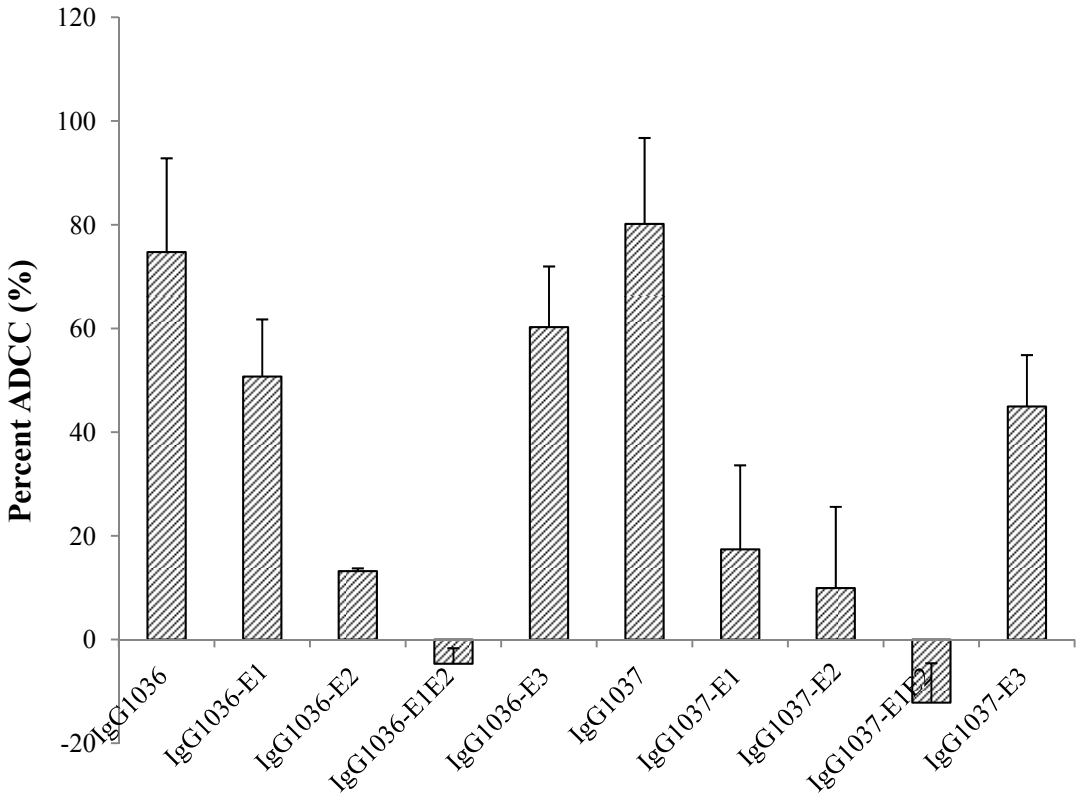
Percent ADCC (%)
120
100
80
60
40
20
0
-20
IgG1036
IgG1036-E1
IgG1036-E2
IgG1036-E1E2
IgG1036-E3
IgG1037
IgG1037-E1
IgG1037-E2
IgG1037-E1E2
IgG1037-E3

TABLE 1 Average AA frequency of three immunodominant epitopes on H1N1 HA.

HA Epitopes	AA position	AA Sequence	Average AA Frequency		
			ADCC++ (M1036 and M1037)	ADCC+ (M1024, M1017 and M1039)	ADCC- (M1089)
HA-E1	92-117	SWSYIVETSSSDNGTCYPGDFIDYEE	51.86±3.16	38.21±0.72	24.11±2.55
HA-E2	124-159	SVSSFERFEIFPKISSWPNHESNKGVTAACPHAGAK	20.66±2.23	32.14±2.93	10.25±1.19
HA-E3	470-521	LKNNAKEIGNGCFEFYHKCDNTCMESVKNGTYDYPKYSEEAKLNREEIDGVK	15.57±1.45	6.54±1.17	40.17±4.09

TABLE 2 Sequence conservation of E1 and E2 in H1N1 strains and all subtypes of influenza A viruses (IFAs) circulating in 2007, 2008 and 2009. In parenthesis: number of HA sequences included in the sequence analysis.

	AA position	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	**Average rate**
	AA sequence	S	W	S	Y	I	V	E	T	S	S	S	D	N	G	T	C	Y	P	G	D	F	I	D	Y	E	E	
HA-E1	**2007_H1N1 (1,000)**	100	100	100	100	100	94	100	67	8	1	8	2	100	100	98	100	100	100	100	2	100	5	98	100	100	100	**76**
	2008_H1N1 (408)	100	100	100	100	99	93	100	13	11	1	13	6	100	100	96	100	100	100	100	7	100	8	94	100	99	100	**75**
	2009_H1N1 (1,000)	100	100	100	100	100	100	100	81	80	80	81	76	100	100	100	100	100	100	100	81	100	81	98	100	100	100	**95**
	2007_IFA (2,325)	33	98	64	64	65	87	100	16	29	13	4	1	63	44	25	100	100	100	67	38	50	2	79	81	67	64	**56**
	2008_IFA (1,339)	41	98	65	65	67	89	100	7	32	10	6	2	63	51	36	100	100	100	68	39	54	3	77	84	68	65	**57**
	2009_IFA (7,880)	86	100	90	90	90	97	100	79	87	78	79	77	89	87	85	100	100	100	90	87	88	78	94	97	90	89	**89**

	AA position	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	**Average rate**
	AA sequence	S	V	S	S	F	E	R	F	E	I	F	P	K	T	S	S	W	P	N	H	D	S	N	K	G	V	T	A	A	C	P	H	A	G	A	K	
HA-E2	**2007_H1N1 (1,000)**	98	100	100	100	99	99	95	100	100	100	100	100	100	1	93	99	100	100	98	100	3	0	2	5	100	97	8	98	8	100	3	95	2	100	7	0	**70**
	2008_H1N1 (408)	96	99	100	100	100	99	97	100	100	98	100	100	99	2	90	100	100	100	96	100	7	0	6	5	100	96	13	96	13	100	6	93	7	100	9	1	**70**
	2009_H1N1 (1,000)	100	100	100	100	100	100	100	100	100	98	100	100	100	81	99	100	100	100	100	100	81	78	81	81	100	99	81	100	81	100	80	100	81	100	81	81	**94**
	2007_IFA (2,325)	68	25	29	31	55	89	37	33	33	54	39	62	51	0	52	48	97	25	33	48	13	5	6	2	100	56	14	25	72	100	30	23	8	52	10	1	**40**
	2008_IFA (1,339)	73	36	37	39	57	89	43	40	40	56	47	62	54	1	52	50	97	38	39	53	10	3	6	3	100	57	14	36	68	100	23	31	10	56	8	1	**42**
	2009_IFA (7,880)	95	85	85	85	89	98	86	85	86	87	87	89	87	77	85	88	99	85	85	87	79	76	78	78	100	87	81	85	94	100	81	84	79	88	79	78	**86**