Supporting Information For

Intracellular delivery of biomineralized monoclonal antibodies to combat viral infection

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Experimental Section

Virus and Cells: IAV H1N1 strain PR8 was grown in 10- to 11-d-old embryonated chicken eggs. DENV-2 strain D2-43 was prepared from supernatants of infected suckling mouse brain suspensions. Purification of the virus was performed by differential centrifugation and sedimentation through a sucrose gradient. Baby Hamster Kidney (BHK-21) cells and Madin-Daby Canine Kidney cells (MDCK) were cultured in DMEM (Invitrogen) supplemented with 10% (vol/vol) FBS at 37°C in an incubator with 5% (vol/vol) CO2.

mAb Biomineralization: mAb solutions (500 μg/mL) were incubated in serum-free DMEM, supplemented with 2-5 mM calcium chloride and 0.15-0.5 mM sodium dihydrogen phosphate, at 37°C for 4 h. The efficacy of biomineralization was determined by measuring the percentage of antibody in the supernatant using the BCA protein kit. The optimized concentrations of 5 mM calcium chloride and 0.375 mM sodium dihydrogen phosphate were used throughout our experiments.

Intracellular delivery of Bm-mAbs: BHK-21 (Baby Hamster Kidney) cells were seeded at a density of 1×10^5 cells per well in a twelve-well plate. Once cells achieved 80% confluence, the medium was replenished with an aqueous solution containing Bm-Alexa Fluor 488-Anti-E, Alexa Fluor 488-Anti-E, and the irrelevant Hab mAb (Human Immunoglobulin monoclonal antibody), respectively (antibody concentrations equaled 200 μg/mL). Intracellular delivery of the Bm-mAbs was achieved by incubation for 4 h at 37°C. The cells were then washed three times with PBS before being directly examined by fluorescence microscopy and further analyzed by flow cytometry. A treatment similar to the above was used on several other mAbs, which were then assessed for their effectiveness in cytosolic delivery within live cells.

Intracellular inhibition of virus replication by Bm-mAbs in cells with established infections: Confluent BHK cell monolayers on nitrocellulose filters were infected with Dengue virus (0.1 M.O.I) via the apical surface for 2 h at 37°C. Diluted aliquots of mAbs containing either Anti-E mAb, Bm-Anti-E mAb, Anti-NS1 mAb, Bm-Anti-NS1 mAb or Hab mAb were added to the lower chamber. After additional 4 h incubation the monolayers were washed with PBS. The monolayers were then incubated for another 48 h at 37°C, at which time the apical supernatants were collected and tested for the presence of virus by plaque assay. The influenza virus was subjected to a similar treatment as above, wherein Bm-mAbs and mAbs were tested for their ability to intracellularly inhibit virus replication.

Plaque Assays: Ninety percent confluent BHK/MDCK cells in a 12-well plate were infected with 400 μL of serial 10-fold viral dilutions. After 1 h of adsorption, the infected cells were washed and incubated for 3-4 d with DMEM containing 2% (vol/vol) FBS and 1% (wt/vol) low-melting-point agarose. The cells were fixed with 4% formaldehyde and stained with crystal violet solution [1% (wt/vol) crystal violet, 0.85% (wt/vol) NaCl, 2% (wt/vol) formaldehyde].

Plaque-reduction neutralization test (PRNT50): Bm-Anti-E mAbs and untreated mAbs were tested with PRNT50 assay for neutralizing antibody. Briefly, Bm-mAbs and untreated mAbs were Serial five-fold dilutions in serum free DMEM. An equal volume of DENV, calculated to yield

approximately 50–100 plaque-forming units (pfu)/0.1 mL, was added to each mAbs dilution and the mixture held 1h at 37°C. Then the mixtures were inoculated into with a confluent layer of BHK-21 cells and placed in a 37°C incubator with 5% CO2 for approximately 1 h. Inoculated wells were then overlaid with DMEM containing 2% (vol/vol) FBS and 1% (wt/vol) low-melting-point agarose. The neutralizing antibody titer was expressed as the reciprocal of the highest initial mAbs dilution that inhibited 50% or greater of the plaque formation compared with the virus control titration.

TEM Characterizations: The Bm-mAbs solutions were added onto carbon coated copper transmission electron microscopy (TEM) grids (400 mesh; Agar Scientific) by dip-coating, and the samples were then dried at room temperature before observation. TEM observations were performed using a JEM-1200EX microscope (JEOL). Samples were prepared by dispersing 50 µL of solution on the surface of silica specimen stubs. They were dried at 30°C for at least 24 h and were sputter-coated with gold before the examinations.

Flow cytometry analysis: BHK cells were seeded at 5.0×10^5 cells per 60 mm tissue culture dish on the day before intracellular delivery. Bm-mAbs complex suspension prepared from serum free DMEM containing 500 µg/mL of mAbs, 0.2 mL Bm-mAbs or untreated mAbs was added into the cells. After 4 h of adsorption, then removal of residual complexes, the cells were collected with 0.25% trypsin, 1.0 mM EDTA solution and washed with 5 mM EDTA solution and centrifugation. Additionally, the cells were washed again with PBS, centrifuged and fixed with 4% (v/v) formaldehyde solution. Before flow cytometry analysis, the cells were washed with PBS, centrifuged and then analyzed with EPICS XL flow cytometer (Beckman Coulter)

Dot Blot Assays: For immunological detection of Bm-mAbs, the untreated and Bm-mAbs were spotted onto a 100% methane-activated PVDF membrane (Millipore) and then air-dried at room temperature. Nonspecific binding sites were blocked using 5% skim milk in PBS. The membrane was then probed with a polyclonal antibody specific to E protein, followed by an alkaline phosphatase-conjugated horse anti-mouse antibody. Both antibodies were diluted in blocking solution (0.05% BSA).

ELISA: Detection of the binding of antibody and antigen , the Bm-mAbs and untreated mAbs was performed by indirect ELISA using 96-well,flat-bottomed plates (Costar) coated with virus .The virus were diluted 1:100 in 0.1 M carbonate/bicarbonate buffer (pH 9.6) overnight at 4°C. After blocking with 5% skim milk powder in PBS and Tween 20 (PBST), plates were incubated with mAbs or Bm-mAbs in duplicate wells for 1 h at 37°C. HRP-conjugated goat anti-mouse IgG at a dilution of 1:5,000 was then added and incubated for 0.5 h at 37°C, followed by the substrate TMB. Plates were washed with PBST (pH 7.2) three times with an interval of 5 min after each reaction. Absorbance of the color developed was determined at 450 nm and corrected for background with PBS control group sera.

Western Blot: Cell lysates were separated on a 12% SDS-PAGE, and the proteins were then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane and blocked 1 h at room temperature in blocking buffer [1x Tris-buffered saline (TBS)−0.1% Tween 20, 5% w/v nonfat dry milk]. Membranes were incubated overnight at 4°C with primary antibodies. All primary antibodies were diluted in dilution buffer [1x Tris-buffered saline (TBS) −0.1% Tween 20, 5% w/v BSA]. The PVDF membranes were extensively washed and incubated with a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (1:2000 dilutions, Rockland, Inc.) for 1 h at room temperature. Proteins were visualized by the ECL method (Pierce).

Immunofluorescence and Confocal Microscopy: Immunofluorescence staining of cells or frozen tissue sections was performed as follow. Briefly, cells were cultivated on coverslips for 24 h and subsequently incubated with mAbs or Bm-mAbs for 4 h at 37°C. Next, the cells were rinsed in PBS solution, fixed in 4% paraformaldehyde (Sigma) in PBS solution for 30 min at 4°C. After two washings with PBS solution, the coverslips were permeabilized in PBS solution containing 0.2% Triton X-100 for 20 min. Then, cells were blocked with 10% normal goat serum for 30 min and stained with secondly antibodies in PBS solution with 0.05% Tween 20 with 3% BSA for 1 h, followed by Alexa Fluor 488-or Alexa Fluor 594-conjugated anti-IgG antibodies of the corresponding species in blocking buffer. After each step, cells were washed at least three times with PBS solution containing 0.05% Tween-20. Coverslips were mounted on slides with ProLong

antifade reagent (Molecular Probes) and examined by using a Zeiss LSM 510 confocal fluorescence microscope. The images were processed by using LSM Image Examiner software (Zeiss).

Animal Test: The animal experiments were approved by the Experimental Animal Ethic and Welfare Committee of Beijing Institute of Microbiology and Epidemiology. All mice were kept under specific pathogen-free (SPF) conditions. The mice (seven per group) were intranasally challenged with a lethal dose (10LD50) of CA07 virus to establish the infection. Then, the treatment by using 200 μL (200 μg/mL) of native Anti-NP or Bm-Anti-NP mAb was applied via intraperitoneal injection and the administration was continued for 3 days. The administration of PBS was defined as the control group. Mice were monitored for 21d for illness and death. Body weight changes were recorded on a daily basis.

Supplementary Figures

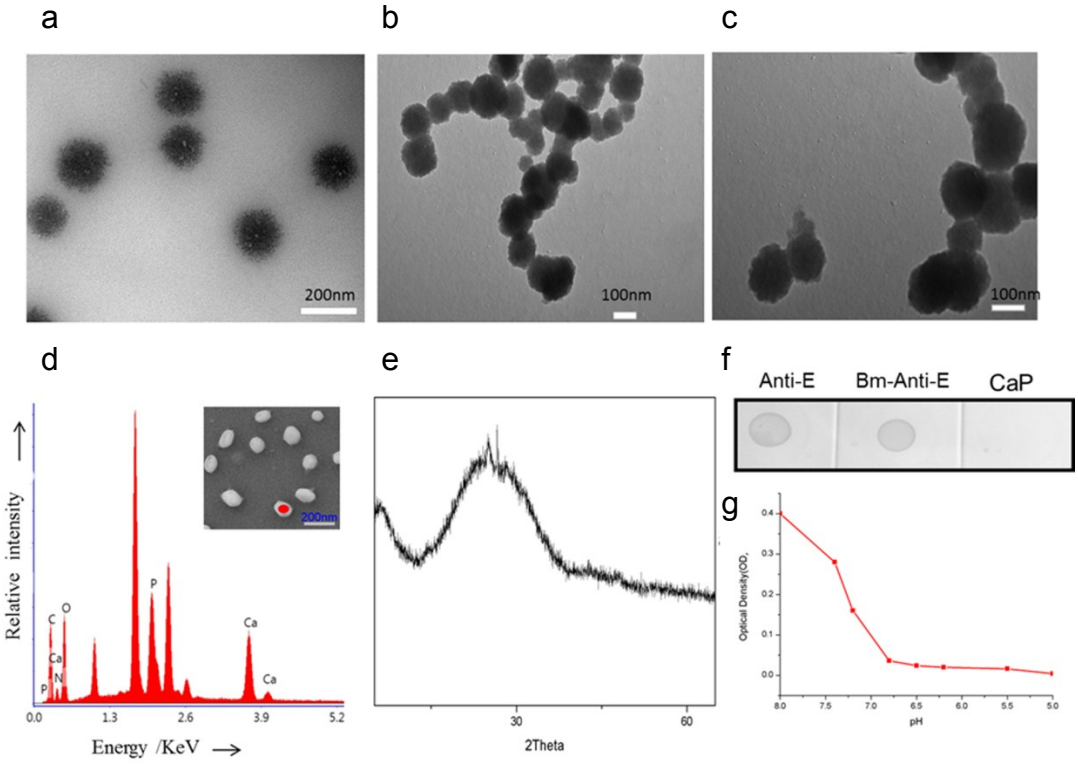

Fig S1. Direct TEM observation of Bm-Anti-HA. (a) Bm-Anti-NP. (b) Bm-Anti-NS1. (c) mAb particles. (d) SEM-EDX analysis of Bm-mAbs, the red indicates the selected area for SEM-EDX. (e) X-ray diffraction analysis of biomineralized mAbs. The pattern indicates that the resulted CaP mineral phase in Bm-mAbs was amorphous. (f) Binding capacity of Anti-E before and after biomineralization, as measured by dot blot assay. (g) Stability curve of Bm-mAbs vs solution pH.

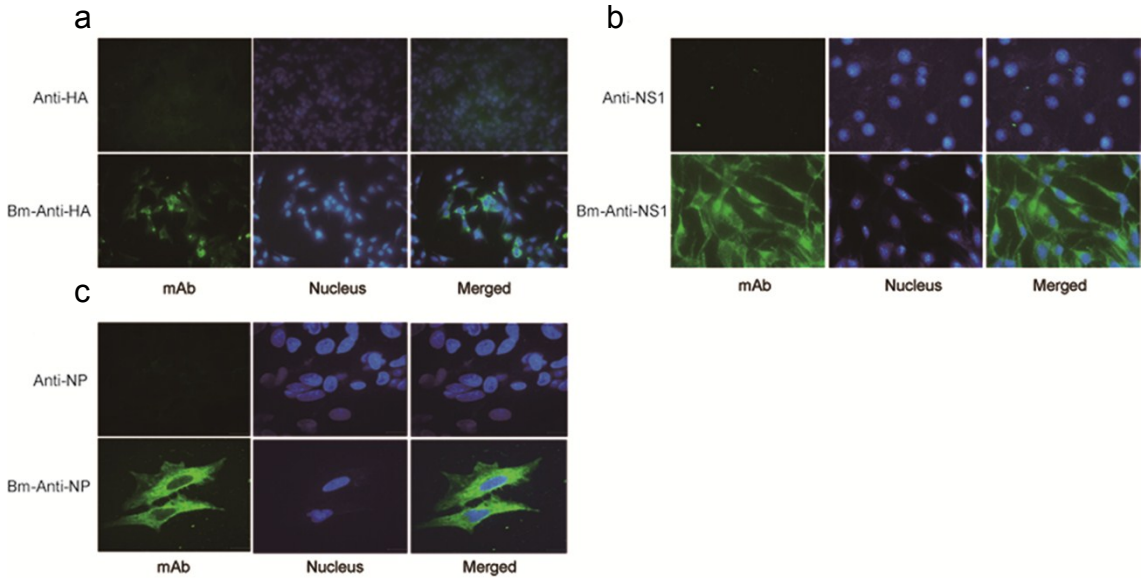

Fig S2. Intracellular delivery of Bm-mAbs to cells. (a) Bm-Anti-HA mAbs in BHK cells. (b) Bm-Anti-NS1mAbs in BHK cells. (c) Bm-Anti-NP mAbs in MDCK cells. Alex-Fluor 488 (green) indicates the mAbs, DAPI (blue) indicates the nucleus.

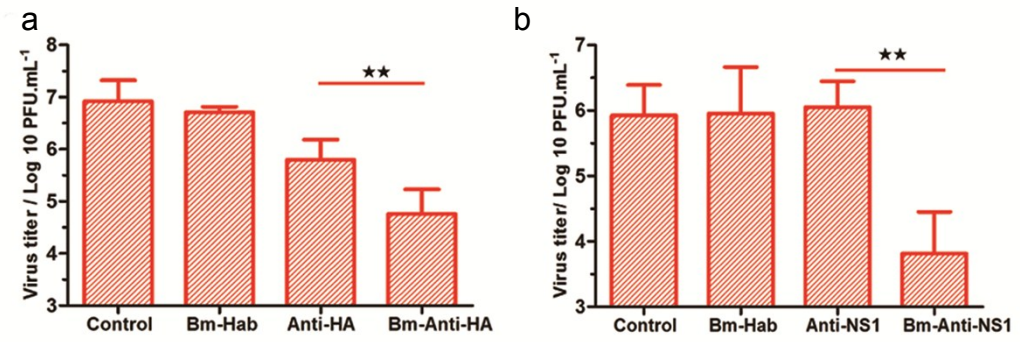

Fig S3. Intracellular inhibition of viral replication by Bm-mAbs. (a) IAV particles were incubated with a monolayer of MDCK cells (M.O.I.=0.1) in the apical chamber at 37°C for 1 h, and then an equivalent quantity of either untreated mAbs or Bm-mAbs (200 μg/mL) was added to the basolateral chamber and incubated at 37°C for 4 h; following this, a PFU test was conducted. (b) DENV particles were incubated with a monolayer of BHK-21 cells (M.O.I.=0.1) in the apical chamber at 37°C for 2 h, and an equivalent quantity of either untreated mAbs or Bm-mAbs (200 μg/mL) was added to the basolateral chamber and incubated at 37°C for 4 h. Error bars represent SDs ($n \geq 3$, Student's paired t test, one-tailed, $^{**}P < 0.01$).

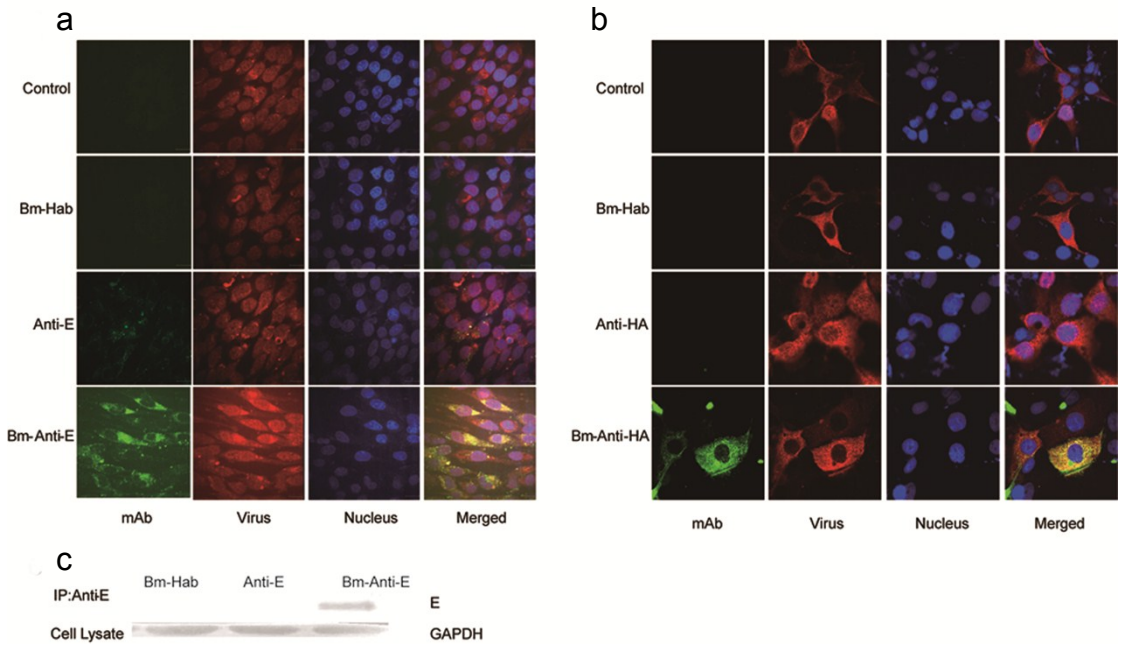

Fig S4. Mechanism of intracellular viral inhibition by Bm-mAbs. (a) Colocalization of Anti-E and DENV in BHK cells. Anti-E mAbs were stained green with Alexa Fluor 488, DENV particles were stained red with Alexa Fluor 594, and the nucleus was stained blue with DAPI. (b) Colocalization of Anti-HA and IAV in MDCK cells. Anti-HA mAbs were stained green with Goat anti-human FITC, IAV particles were stained red with Alexa Fluor 594, and the nucleus was stained blue with DAPI. (c) Co-Immunoprecipitation (Co-IP) assays to evaluate interactions between the antibody and the protein.

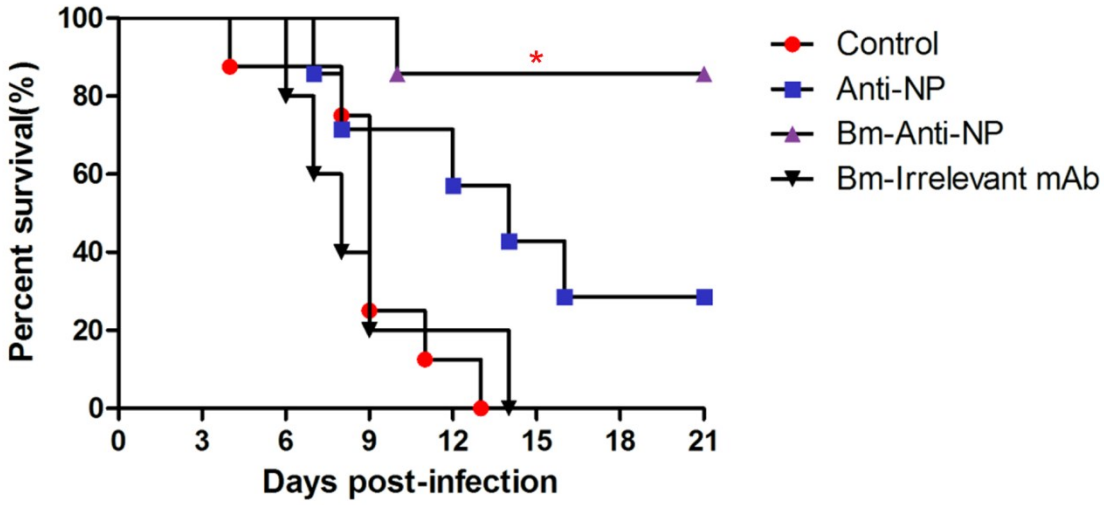

Fig S5. Survival rate was assessed by recording whether the mice died from the infection using Bm-mAbs and mAbs after challenge. (* means P< 0.05 for Bm-Anti-NP group vs. Anti-NP group, Student's paired t test).

Supplementary Table

Table S1 Four kinds of anti-infective monoclonal antibodies in our study.

Virus	mAb	Target	Development technology	Neutralization Antibody	Reference
Dengue	Anti-E	E protein	Murine	Yes	6
Dengue	Anti-NS1	Non-structure protein	Murine	No	3
Influenza	Anti-NP	Nucleoprotein	Murine	No	35
Influenza	Anti-HA	Hemagglutinin	Humanized	Yes	7