Supplemental materials for: Dai et al

Influenza virus culture

Influenza virus strain A/PR/8/34 was purchased from ATCC, as were the MDCK cells in which the virus was propagated. MDCK cells were split and cultured overnight in RPMI 1640 with L-glutamine (Invitrogen, Singapore) supplemented with 10% foetal bovine serum (Gibco, Invitrogen, Singapore), to reach approximately 50% confluence. First the cells first were washed with PBS, then the virus was added to the cells, and freshly prepared media was then added. The cells were then incubated at 33°C with 5% CO_2; the supernatant was harvested when cell lysis was widespread. The virus was concentrated using the 100,000 MW Vivaspin 20 centrifugal concentrators (Sartorius AG, Singapore).

Haemagglutination assay to quantify viral titres

Serial dilutions of the Influenza A virus were prepared. 50µl of 0.5% guinea pig red blood cell solution was then added to each well. The plate was incubated at 4°C overnight and viewed the following day to determine the haemagglutination assay titre (HA) of the virus stock.

Generation of human anti-influenza antibodies

Human volunteers were immunized with the seasonal influenza vaccine, and blood was obtained 21 days after vaccination. The serum was then run through a 5ml HiTrap Protein G HP column (GE Healthcare) on an ÄKTAFPLC fast protein liquid chromatography machine (GE Healthcare) on the UNICORN 5.10 platform. The eluted hIgG was adjusted to pH7 using 1M Tris-HCl pH 9.0. The antibody obtained was then tested for reactivity against the A/PR/8/34 strain of influenza that was used in the experiments, using the haemagglutination inhibition assay.

Haemagglutination inhibition assay to quantify neutralising antibody titre

Following RBC blocking for non-specificity, each hIgG sample was serially diluted in a 96-well round-bottom plate at 25µl of hIgG/well. 4 HAU of A/PR/8/34 was added to each well. The plate was incubated at 4°C for 1 hour. 50µl of 0.5% RBC solution was then added to each well. The plate was incubated overnight at 4°C. The plate was read, and the dilution at which the red ring of RBC disappears was taken to be the dilution factor of the hIgG required to neutralize 4 HAU of virus.

Generation of immune complexes composed of influenza A and Human IgG

An excess of hIgG was incubated with A/PR/8/34 for 2 hours at 4°C. The mixture was then loaded into two 1,000,000 MW Vivaspin 2 centrifugal concentrators (Sartorius AG, Singapore) and centrifuged for 90 minutes at 3,000 g at 4°C. The top fraction containing the immune complex was removed from each concentrator, pooled together, and diluted to a total volume of 400µl. Purified IC was incubated at a 1:5 dilution per 1×10^6 primary human monocytes for 10mins at 37°C in a total volume of 200ul in serum free medium. Unbound ICs were washed off in serum free medium (300G, 7mins, RT).

Confocal microscopy

Cells were fixed on poly-L-lysine-coated coverslips using a mixture of methanol and acetone. They were incubated with primary antibodies targeting EEA-1, TI-VAMP, Syntaxin 4, LAMP-1 (Santa Cruz, USA), and HLA-DM respectively (Provided by Dr.Adrian Kelly, Cambridge University, UK) for 2 hours, then stained with Alexa Fluor-conjugated secondary antibodies (Invitrogen, Singapore) and DAPI for 1 hour.

Cells were washed and mounted on slides. The slides were viewed either on a Zeiss LSM510 confocal microscope or on a Leica SP5 confocal microscope at 63× magnification.

Generation of primary influenza a specific human T-cell line

$CD14^+$ monocytes were isolated from healthy human volunteers and resuspended in 1 ml of DMEM without serum. 200 HAU of the A/PR/8/34 strain of influenza virus was added and incubated at 37°C for 2 hours. $CD14^+$ cells were washed and resuspended in DMEM media and added onto $CD4^+$ cells, cultured in a mix of DMEM with 10% human AB serum and 10 U/ml human IL-2 (PeproTech). $CD4^+$ cells were re-stimulated with fresh $CD14^+$ monocytes infected with A/PR/8/34 on Day 7 and used for the proliferation assay on Day 14.

Files in this Data Supplement:

Figure S1. Preparation of primary human monocytes for FcR binding studies (JPG, 160 KB) -
(A) Flow cytometric analysis of surface hIgG on unfractionated primary monocytes from human PBMNC. i) PBMNC labelled with goat anti-human $F(ab')_2$ FITC(Thermo Scientific, USA) and anti-CD14PE (BD Pharmingen) for 20mins at 4°C prior to washing ×2 in ice cold PBS and fixation in 3% Paraformaldehyde. Dot plot analysis of surface hIgG on CD14 gated cells based on percent staining in upper right quadrant. ii) Surface hIgG staining on purified human monocytes washed in ice cold citrate buffer (PBS/0.4% Sodium Citrate) and cultured in serum-free medium. iii) Surface hIgG staining on purified monocytes pre-incubated with 10µM purified monomeric hIgG (Sigma-Alldrich) for 10mins at 4°C. (B) Purified human monocytes (i) were incubated with hIgG (10µM) and labelled as described for flow cytometry without the paraformaldehyde fixation step (ii). Unlabelled IC were added at volume ratios of (iii)1:10, (iv)1:5 and (v)1:1 in serum free medium from a purified stock and displacement of labelled Monomeric hIgGs on the surface of the monocytes over 10minutes at 37°C assessed as a predicted reduction in surface fluorescence.

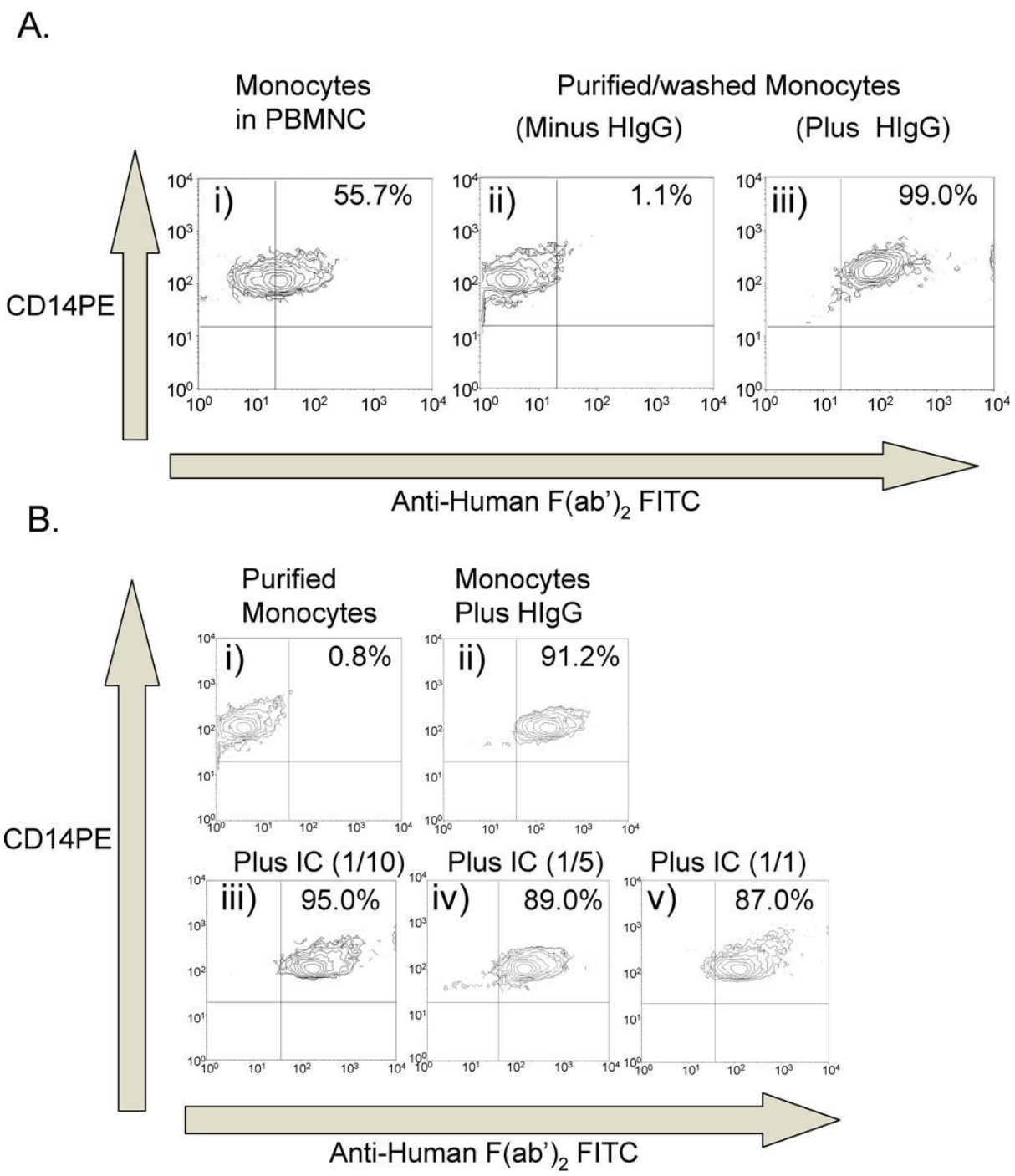


Figure S2. Differential membrane trafficking of Fcγ receptors (JPG, 546 KB) -

(A) U937 cells were cross-linked with either anti-FcγRI (antibody 10.1) and/or anti-FcγRII (antibody 3D3) for 10 minutes, then fixed and stained for SNARE proteins that aid in vesicular trafficking. Both FcγRI and FcγRIIa enter a compartment that stains positive for (i) Syntaxin 4 and (ii) TI-VAMP. (B) FcγRI and FcγRII in primary dendritic cells and monocytes show similar trafficking patterns to U937 cells. The Fcγ receptors on the primary cells were incubated with mouse α-FcγRI and/or α-FcγRII, then cross-linked with

a secondary anti-mouse that had been labelled with an Alexa-Fluor 647 dye. The Fcγ receptors were cross-linked at 37 °C for 10 minutes, and the cells were fixed, then stained for EEA-1 and LAMP-1. In both (i) dendritic cells and (ii) monocytes, FcγRI, and FcγRII enter early endosomes marked by EEA-1. In both (iii) dendritic cells and (iv) monocytes, however, it is observed that, while the compartment containing FcγRI continues to mature into a late endosome marked by LAMP-1, the compartment containing FcγRII does not. (C) Flow cytometric analysis indicates that the level of FcγRIII present on the surface of primary monocytes is minimal. NK cells are used as a positive control as they express high levels of FcγRIII.

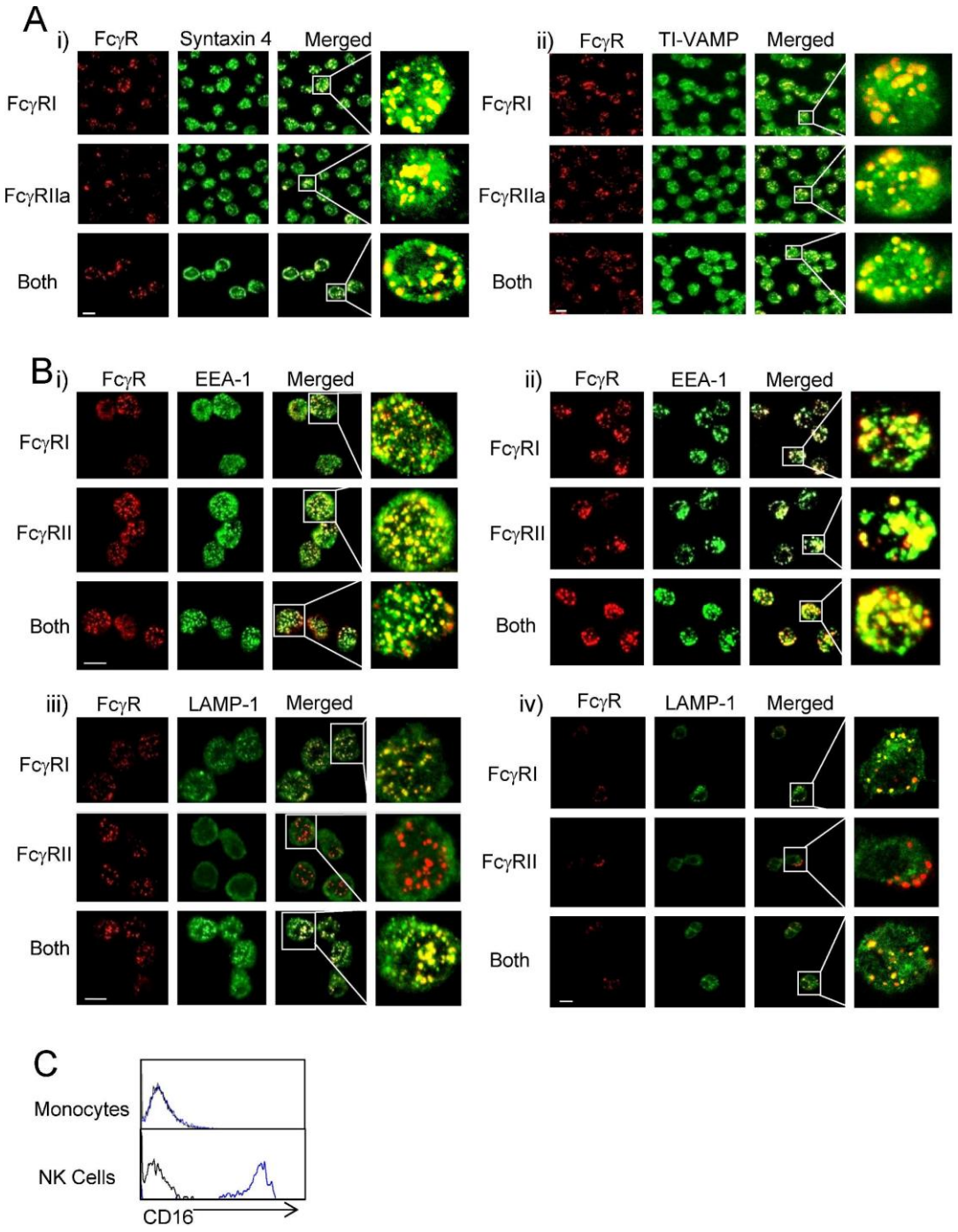
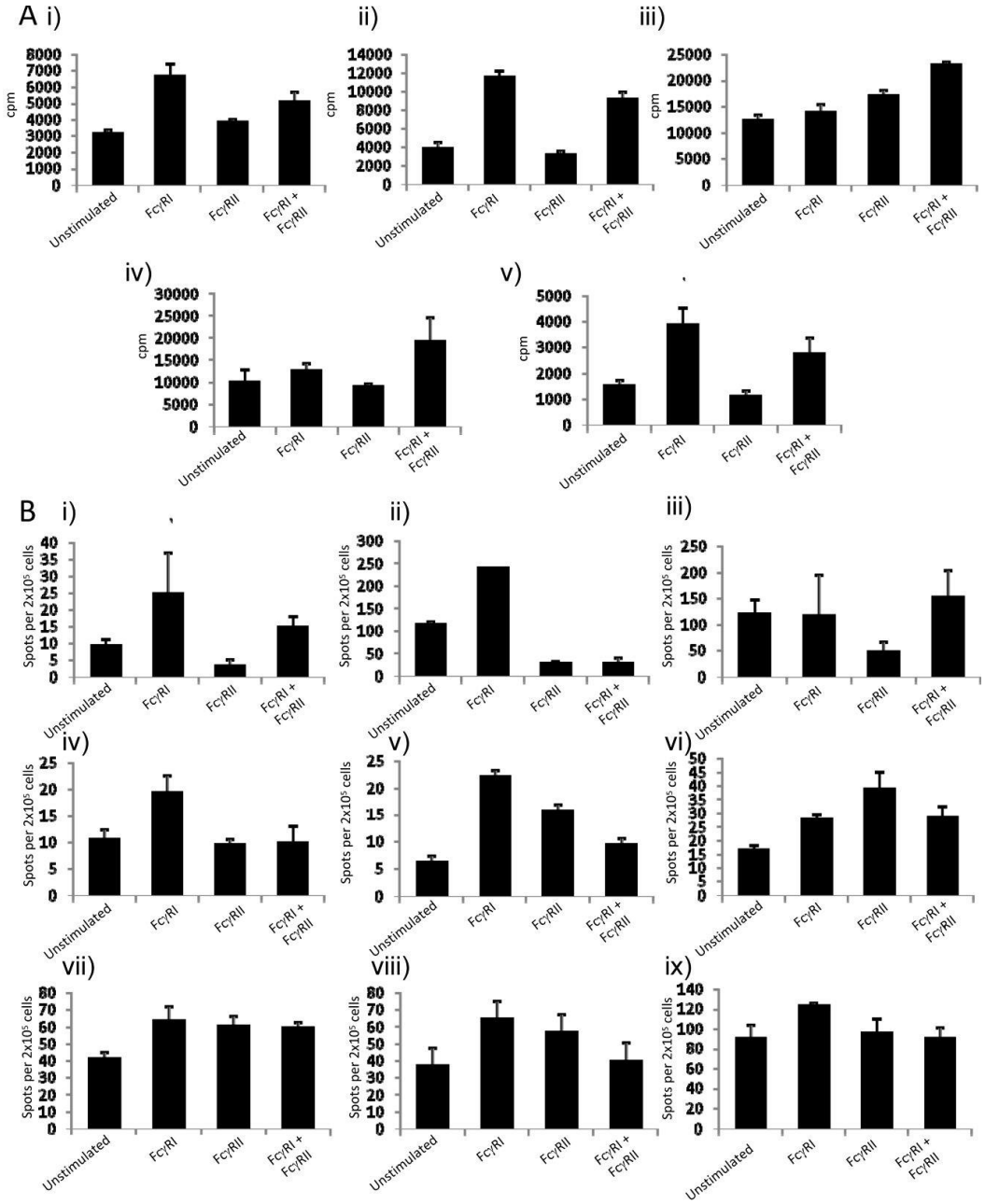


Figure S3. Analysis of influenza A antigen-presentation following internalisation of IC via FcγRI and/or FcγRIIa (JPG, 216 KB) -

(A) Antigen specific proliferation of influenza specific $CD4^+$ T lymphocyte lines determined by 3H-Thy incorporation. Primary monocytes from human donors were pulsed with influenza A/hIgG immune complexes for 10minutes prior to incubation with syngeneic influenza specific human $CD4^+$ T-cell lines for 4-7days. Internalisation of ICs via FcgRI was carried out in the presence of a blocking antibody to FcgRIIa (IV.3). Internalisation of ICs via FcγRIIa was carried out in the presence of excess monmeric human IgG to block FcγRI. Data from 5 independent cell lines is presented as mean cpm of triplicate cultures plus SD (i-v). (B) ELISPOT analyses of influenza A specific CD4 T-cell responses in primary human cells. Monocytes and $CD4^+$ T cells were enriched by CD8 depletion prior to internalisation of IC via FcγRI and/or FcγRIIa. ELISPOTs based on activation of influenza specific CD4 T cells allowed to develop over 14-18hrs in culture prior to quantification using a CTL ImmunoSpot S4 Analyzer. Data from 9 individual experiments in unrelated human donors is shown as the mean number of spots plus SD per 2×10^5 cells in duplicate wells (i-ix).


A i)
ii)
iii)
iv)
v)
cpm
Unstimulated
FcγRI
FcγRII
FcγRI + FcγRII
B i)
ii)
iii)
iv)
v)
vi)
vii)
viii)
ix)
Spots per 2x10⁵ cells