PB1-F2 derived from avian influenza A virus H7N9 induces inflammation via activation of the NLRP3 inflammasome.

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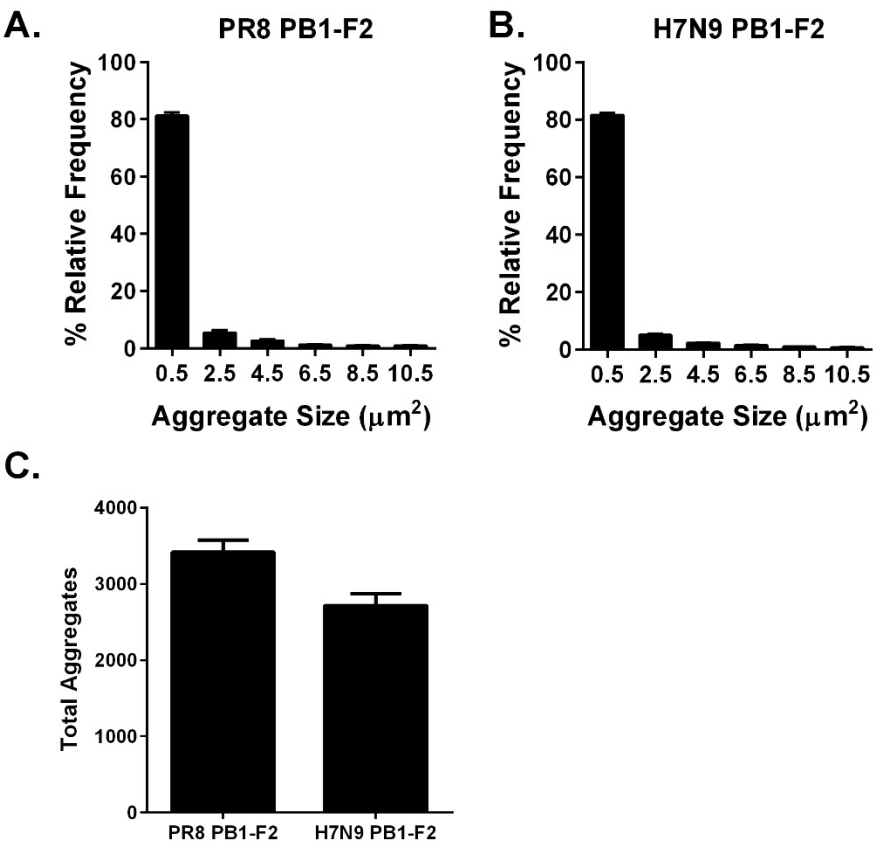
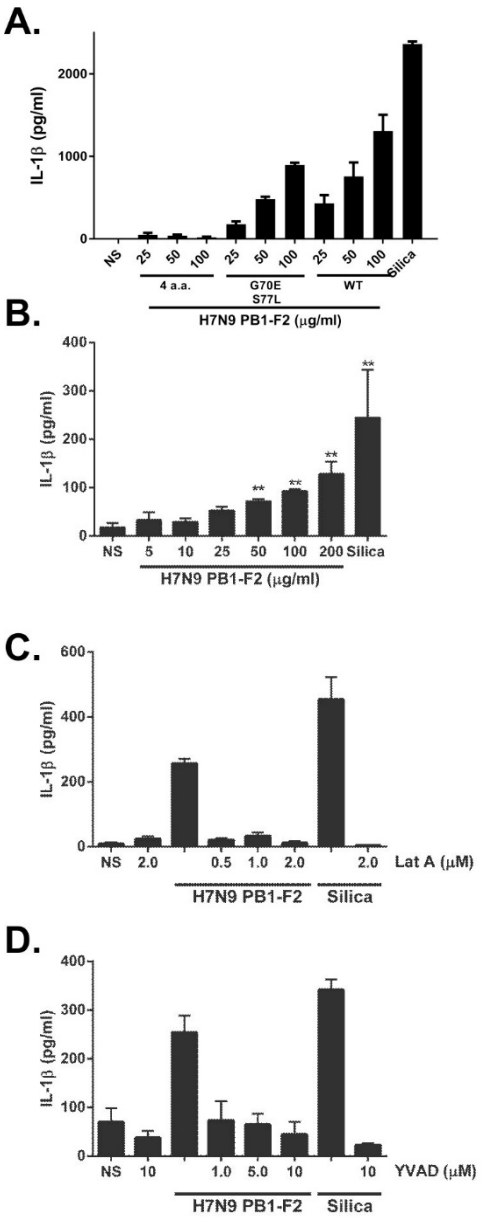
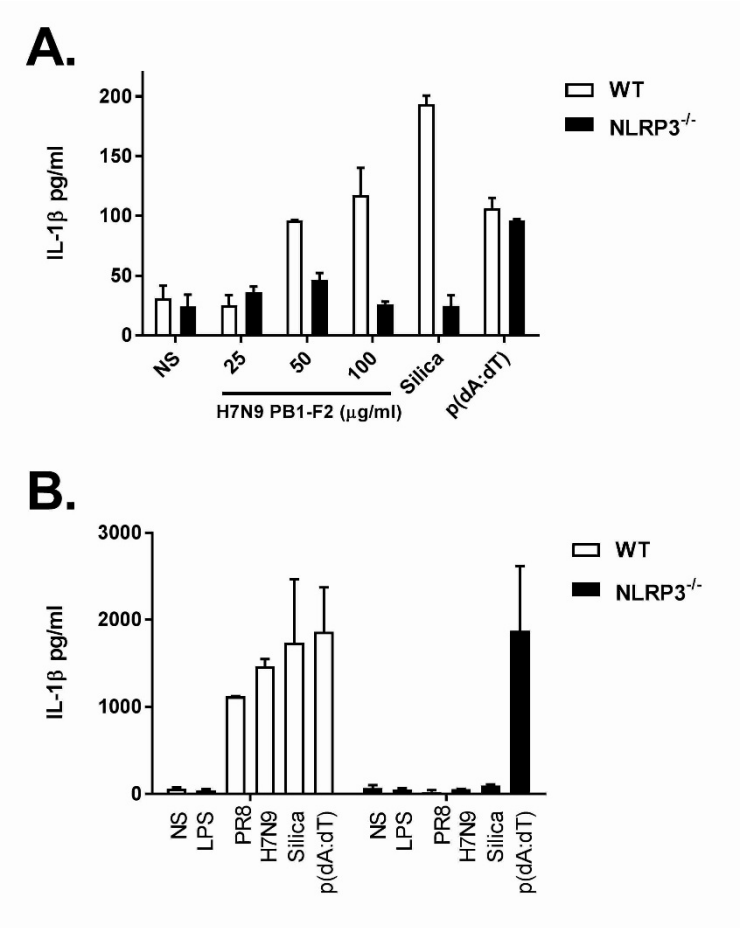


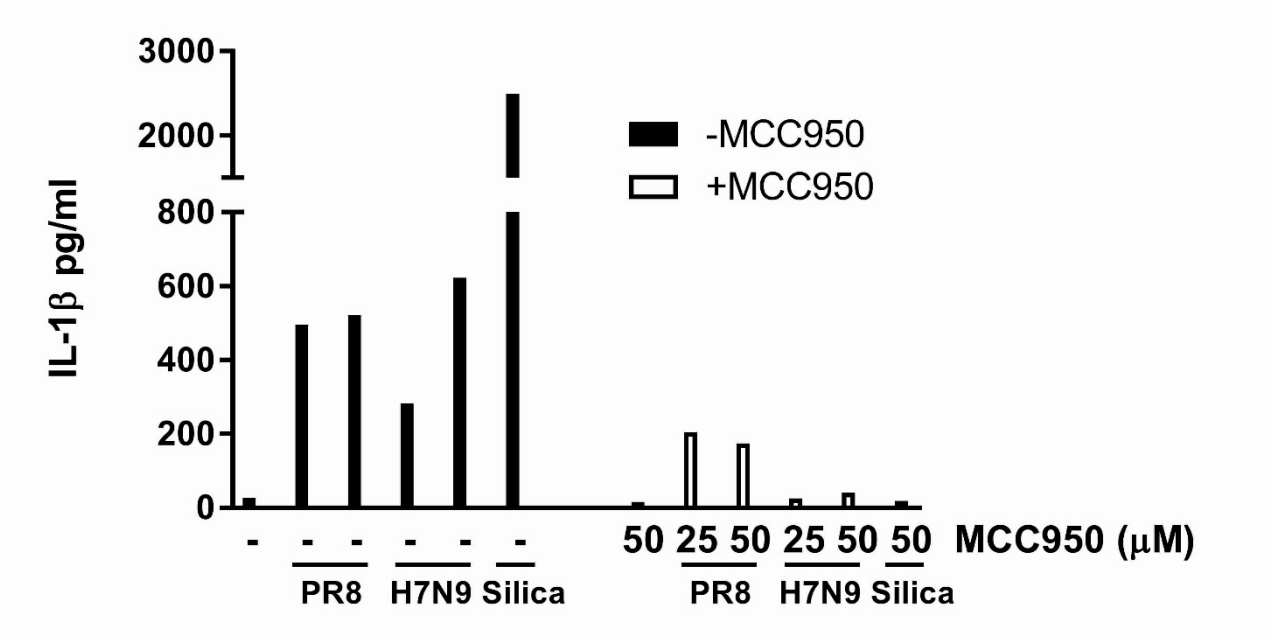
Supplemental Data 1. PR8 and H7N9 PB1-F2 aggregates are the optimal size for inflammasome activation. Five fields of 3 drops (2μl) of each of (A) PR8 and (B) H7N9 PB1-F2 peptides were imaged using the DeltaVision confocal microscope. Peptide aggregates contained with each of the images were analysed using Fiji software for (**A** and **B**) the percentage and frequency of aggregates that are within the optimal size for inflammasome activation (ie 0.5-3 μm in areas)within the PR8 and H7N9 PB1-F2 solutions respectively, and (C) the total number of aggregates contained within the peptide solutions



Supplemental Data 2. H7N9 PB1-F2 induction of the inflammasome is caspase-1- and actin polymerization-dependent. (**A**) Immortalized BMDMs (iBMDMs) were primed with LPS (100ng/ml) for 3 h prior to challenge with a dose-range (25-100μg/ml) of indicated H7N9 PB1-F2 peptides for a further 6 h. (**B**) Primary BMDMs primed with LPS (100 ng/mL) for 3 h were stimulated with the indicated doses of H7N9 PB1-F2 peptide (5-200 μg/mL), silica (150 μg/mL) or left unstimulated (NS) for 6 h. iBMDMs were primed for 3 h with LPS and **(C)** pre-treated with latrunculin A (at doses indicated) or with **(D)** YVAD (at doses indicated) 40 mins prior to stimulation with H7N9 PB1-F2 peptide (100 μg/mL), silica (150 μg/mL) or left unstimulated (NS) for a further 6 h. Cultured supernatants were harvested and examined for secreted IL-1β by ELISA. Results are represented as mean ± SEM and are representative of 3 independent experiments. ** $p < 0.01$, One-way ANOVA



Supplemental Data 3. H7N9 PB1-F2 peptide induces IL-1β in a NLRP3-dependent manner. BMDMs derived from wild-type or NLRP3 deficient mice were primed with LPS (100 ng/mL) for 3 h. **(A)** Cells were subsequently stimulated with a dose range of H7N9 PB1-F2 peptide (25-100 μg/mL), silica (150 μg/mL) or poly(dA:dT) (500 ng/mL) or left unstimulated (NS) for a further 6 h. **(B)** Cells were stimulated with H7N9 PB1-F2 peptide (100 μg/mL), PR8 PB1-F2 peptide (100 μg/mL), silica (150 μg/mL), poly(dA:dT) (500ng/mL) or left unstimulated (NS) for a further 6 h. Cultured supernatants were collected and assayed for the levels of secreted IL-1β by ELISA. Results are presented as mean ± SEM and are representative of 3 independent experiments



Supplemental Data 4. MCC950 inhibits H7N9 PB1-F2-induced IL-1β secretion in macrophages. Primary BMDMs were primed with LPS (100 ng/mL) for 3 h. Cells were then pre-treated with MCC950 at the indicated doses, or not, 40 mins prior to stimulation with the H7N9 or PR8 PB1-F2 peptides (100 μg/mL), silica (150 μg/mL) or left unstimulated (NS) for a further 6 h. Cultured supernatants were then harvested and assayed for the levels of secreted IL-1β by ELISA. Results are presented as mean ± SEM and are representative of 3 individual experiments.

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A/Wuxi/4/2013                     ----------------------------------------------------------------------------------------
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Supplemental Data 4. Alignment of 89 full length PB1-F2 proteins (1-90 amino acids) sequenced from clinical isolates of H7N9 from infected humans. A/Shanghai/2/2013 strain used as the template in our study is the consensus sequence for the alignment. Alignment demonstrates the low divergence amongst H7N9 PB1-F2. Red residues in consensus sequence correspond to the H7N9 peptide used in the study. Importantly, there is no changes amongst the 4 critical inflammatory amino acid resides (blue color) identified in the C-termini of PB1-F2. Sequences obtained from the Influenza Research Database (http://www.fludb.org).

Supplemental Movie 1. Live cell imaging of H7N9 induction of ASC speck formation in macrophages.

Live cell imaging of static images portrayed in Figure 2 Image bar is 8μm.

Supplemental Movie 2. H7N9 induces rapid uptake and ASC speck formation in macrophages.

Live cell imaging of pHrodo-labelled H7N9 PB1-F2 peptide-induced ASC speck formation in macrophages, depicting a wide field of view demonstrating activation of a significant number of cells commensurate with the more detailed view shown in Figure 2 and Movie S1. Image bar is 80μm.

Supplemental Movie 3. Inhibition of phagocytosis blocks uptake of pHrodo-labeled H7N9 peptide and ASC speck formation.

Live cell imaging of wide view of large number of macrophages pre-treated for 40 mins with Latrunculin A (2.0 μM) and then treated with 100μg/mL of pHrodo-labeled H7N9 peptide. Image bar is 100μm.

Supplemental Movie 4. H7N9 PB1-F2 peptide induces mtROS production and subsequent ASC speck formation.

Live cell imaging of wide view of multiple fields of view of ASC-cerulean iBMMs pretreated with MitoSOX (1 μM) for 10 mins prior to challenge with H7N9 PB1-F2 peptide (100μg/ml) for 4 h. Image bar is 100μm.

Supplemental Movie 5. PR8 PB1-F2 peptide induces mtROS production and subsequent ASC speck formation.

Live cell imaging of wide view of multiple fields of view of ASC-cerulean iBMMs pretreated with MitoSOX (1μM) for 10 mins prior to challenge with H7N9 PB1-F2 peptide (100μg/ml) for 4 h. Image bar is 100μm.

Supplemental Movie 6.

Live cell imaging of wide view of multiple fields of view of ASC-cerulean iBMMs pretreated with bafilomycin A (1 μM) for 45 mins and MitoSOX (1μM) for 10 mins prior to challenge with H7N9 PB1-F2 peptide (100μg/ml) for 4 h. Image bar is 100μm.

Supplemental Movie 7. MCC950 inhibits H7N9 PB1-F2-induced ASC-speck formation despite uptake of peptide

Live cell imaging of ASC-cerulean iBMDMs pretreated with MCC950 (50μM) for 10 mins prior to challenge with pHrodo labelled H7N9 PB1-F2 peptide (100μg/ml) for 4 h. Image bar is 5μm.

Supplemental Movie 8. Multiple field view of MCC950 suppressing ASC-speck formation despite uptake of H7N9 PB1-F2 peptide

Live cell imaging of wide view of multiple fields of view of ASC-cerulean iBMMs pretreated with MCC950 (50nM) for 10 mins prior to challenge with pHrodo-labeled H7N9 PB1-F2 peptide (100μg/ml) for 4 h. Image bar is 100μm.

Experimental Procedures

Peptide aggregate analysis

H7N9 or PR8 PB1-F2 peptides were pipetted on a well of an Ibidi 8-well chamber slide. 15 fields were imaged using a DeltaVision confocal microscope (magnification 600x). Aggregates were then analysed using Fiji software to obtain the frequency of aggregates to be within the optimal size range (0.5-3 μm) in area for activating the inflammasome.

MitoTempo live cell imaging

ASC-cerulean iBMDMs were plated at 2x105/ml in Ibidi 8-well chamber slides 24 h prior to stimulation. Cells were pretreated with MitoTempo (20μM) for 45 mins prior to challenge with PB1-F2 peptide (100μg/ml). Cells were imagined via live time-lapse imaging over the time indicated at 37ºC using the API DeltaVision Deconvolution micorscope equipped with temparature and CO_2 controlled sample chamber. Imaging was at 600x magnification. Representative images show the 3D deconvolution of multiple z-stack