SUPPLEMENTAL EXPERIMENTAL PROCEDURES:

Experimental Animals

All animals are maintained on a C57Bl/6 genetic background and all experiments were carried out with age (8-10 weeks old) and sex matched mice. Mice were bred and maintained in specific pathogen-free animal facilities at The University of North Carolina at Chapel Hill.

Virus Propagation

The influenza virus A/Victoria/3/75 (H3N2) is a recombinant virus generated from cloned cDNA in 293T cells and propagated in MDCK cells. Confluent flasks of MDCK cells were inoculated with stock virus at a MOI equal to 0.01 in 2ml serum-free DMEM for 1 hour at 37°C. After incubation, additional serum-free DMEM containing trypsin (Worthington) was added. Flasks were monitored for cytopathic effect and culture supernatants harvested when destruction of the monolayer was observed (3-4 d.p.i.). Supernatants were centrifuged to remove cell debris and stored in aliquots at -80°C.

Viral stock titers and titers in experimental samples were determined by standard plaque assay on confluent monolayers of MDCK cells in 12-well plates. Following incubation at 37°C, inoculum was removed and cells overlaid with 2% agarose (Oxoid) / EMEM with 1μg/ml trypsin (Worthington). The agarose overlay was removed after three days of incubation at 37°C and plaques visualized by methanol fixation and 1% crystal violet stain.

shRNA Knockdown in Human Monocytic Cell Lines

Wild type and mutant human short hairpin (sh) RNAs were stably expressed in the human THP-1 monocyte cell lines by infection with a lentivirus containing shRNA transcription

cassettes driven by the H1 RNA promoter and a separate cassette containing green fluorescent protein driven by the phosphoglycerate kinase promoter. In addition to the specific targeting sequences for NLRP3, ASC and TUCAN, plasmids carrying mutated targeting sequences (negative) and luciferase (positive) were also designed to serve as controls. The generation of these NLRP3 and ASC attenuated THP-1 cells has been previously published (Taxman et al., 2006a; Taxman et al., 2006b; Willingham et al., 2007). To verify successful knockdown of the desired NLR, total RNA was isolated from cell pellets using Trizol reagent (Invitrogen) following the manufacturer's protocol. Real-time PCR was performed with the TaqMan sequence detection system and commercially available primer/probe sets for each NLR (Applied Biosystems). All results are normalized to 18s ribosomal RNA and data was analyzed using the comparative C_T method ($\Delta\Delta C_T$).

Human airway tracheobronchial epithelial cells were derived from excess tissue following lung transplantation and provided by the UNC Cystic Fibrosis Center Tissue Culture Core under The UNC-CH IRB-approved protocols and cultured as previously described (Bartlett et al., 2008). Human airway epithelial cell lines (JME) were cultured as previously described (Jefferson et al., 1990).

Real-time PCR Expression

Total RNA was isolated from frozen homogenized lung tissue pellets utilizing Trizol reagent (Invitrogen) following the manufacturer's protocol. Total RNA was reverse transcribed using a High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's protocols. Lung cDNA was amplified using Taqman PCR Universal Master Mix (Applied Biosystems) and the Applied Biosystems 7900 HT Fast Real-Time PCR Platform using

commercially available primer/probe sets (Applied Biosystems). All experimental samples were run in triplicate and the relative expression was determined by normalizing samples to the *18S rRNA* housekeeping gene. Data was analyzed using the comparative C_T method ($\Delta\Delta C_T$).

SUPPLEMENTAL DATA:

S1. Increased morbidity is associated with Influenza A/PR/8/34 infection. Wild type (C57Bl/6), $Asc^{-/-}$, $Nlrp3^{-/-}$ and $Nlrc4^{-/-}$ mice were challenged intranasally (i.n.) with 6×10^4 PFU/ml of mouse adapted Influenza A/PR/8/34 and morbidity (weight loss) was monitored throughout the course of infection. All mice demonstrated significant weight loss through day 7 of this model, with no significant differences identified between genotypes. Wild Type (n = 4); $Asc^{-/-}$ (n = 6); $Nlrp3^{-/-}$ (n = 5); $Nlrc4^{-/-}$ (n = 3).

S2. *Ex vivo* bone marrow derived macrophages do not exhibit robust influenza A virus replication. Bone marrow derived macrophages were harvested from mice following standard protocols. (**A**) Macrophages of various genotypes were challenged with influenza A/Victoria/3/75 (MOI = 1) and cell free supernatants were harvested over a time course. Enhanced viral clearance and a precipitous drop in viral titer were observed in macrophages of all genotypes 12 hours post-challenge. For a comparison, a robust viral replication was observed in human pulmonary macrophages infected with the influenza A/Victoria/3/75 strain (filled square). **(B)** Nlrp3 and ASC did not affect type I IFN production. Increased levels of IFN-β were observed 12 hours following viral challenge and this process was not dependent on ASC or Nlrp3.

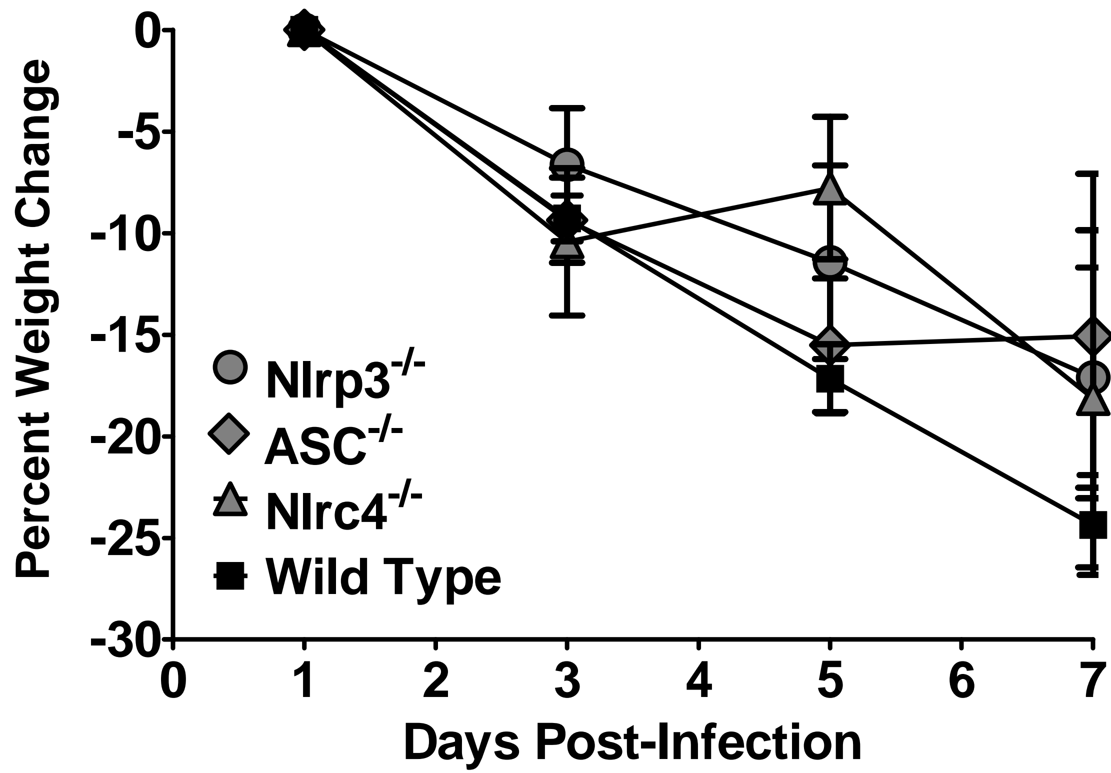
S3. Mouse Airway Epithelial Cells and Macrophages are the predominant cell types infected by influenza virus A/PR/8/34 (H1N1) *in vivo*. To identify the cell types of highest *in vivo* relevance to influenza infection in mice, C57Bl/6 mice were challenged intranasally (i.n.) with 6 X 10^4 PFU/ml of a mouse adapted influenza virus A/PR/8/34. The lungs were harvested 3 days post-infection, fixed in 10% buffered formalin and paraffin embedded for histology. To assess infected cell types in the mouse lung, tissue sections were deparaffinized and subjected to IHC utilizing standard procedures and a FITC conjugated goat polyclonal antibody specific to influenza A H1N1 viruses (Abcam Inc, MA). Sections were stained with FITC labeled goat polyclonal anti-influenza A virus antibody and visualized with DAPI staining (not shown) in VectorShield mounting medium under confocal microscopy. Approximately 15% of the macrophages present in the lungs were IHC positive for viral antigen. **(A)** Representative small airway demonstrating high numbers of airway epithelial cells which were FITC positive for viral antigen. **(B)** Representative FITC positive airway epithelial cells are shown. **(C)** DIC image of **(B). (D)** Representative FITC positive macrophage, within an alveolar space, is shown from an influenza virus-infected wild type mouse. **(E)** DIC and **(F)** FITC/DIC overlay image of the macrophage shown in **(D)**. **(G–I).** Influenza-negative macrophages and other leukocytes were also observed in lungs from these animals. **(G)** Representative FITC negative macrophage located within an alveolar space. **(H)** DIC and **(I)** FITC/DIC overlay of the macrophage shown in **(G)**. Data are representative of 10 individual mice assessed in 2 independent experiments.

S4. Reduced IL-1β protein is not due to reduced transcription. Nlrp3- and ASC-deficient mice were analyzed for IL-1β transcript expression. Whole lungs were harvested, weighed and homogenized from influenza and mock inoculated animals. Total RNA was extracted and real

time PCR data indicate that the decrease in IL-1β protein is not at the level of transcriptional control, as increased levels of IL-1β transcript were observed in lungs from Nlrp3$^{-/-}$ and Asc$^{-/-}$ mice. Samples were normalized to 18s and compared to target gene expression in mock inoculated animals.

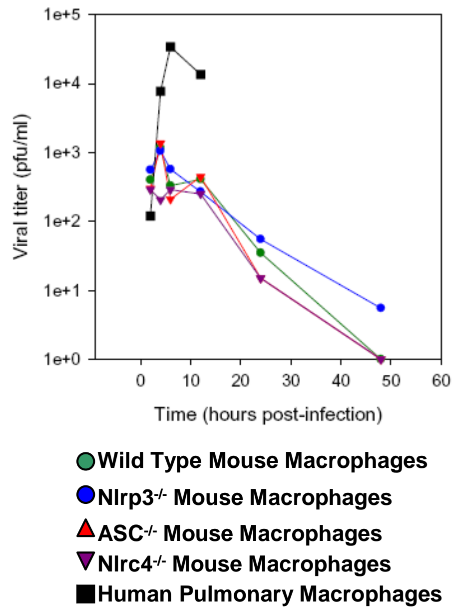
S5. Successful generation of ASC, NLRP3 and TUCAN knockdowns in human monocytes. A-C) ASC, NLRP3 and TUCAN were knocked down in human THP-1 cells by infection with a lentivirus containing shRNAs for the targeted gene (shASC, shNLRP3 or shTUCAN) or containing a targeting cassette with a mutated sh target sequence (shmut). Confirmation of gene reduction by shRNA was assessed by real-time quantitative PCR normalized to 18s. **(D)** Cells bearing shRNA and unmanipulated THP-1 were infected with A/Victoria/3/75 for 2 hours at an MOI = 1 and show equal viral titer in the supernatant at 24 hours post-infection. **(E)** Increased IL-1β transcription was observed in the wild type THP-1 cells 24 hours post-infection with A/Victoria/3/75. **(F)** Influenza virus mediated release of TNFα in THP-1 cells is independent of NLRP3 and ASC activity. Knockdown experiments are representative of at least 3 independent experiments.

Supplemental Figure S1.

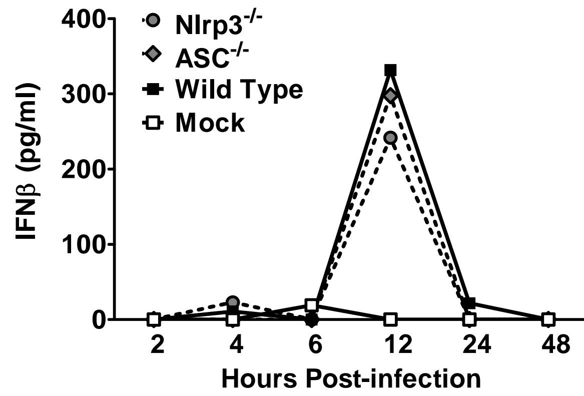


Supplemental Figure S2.

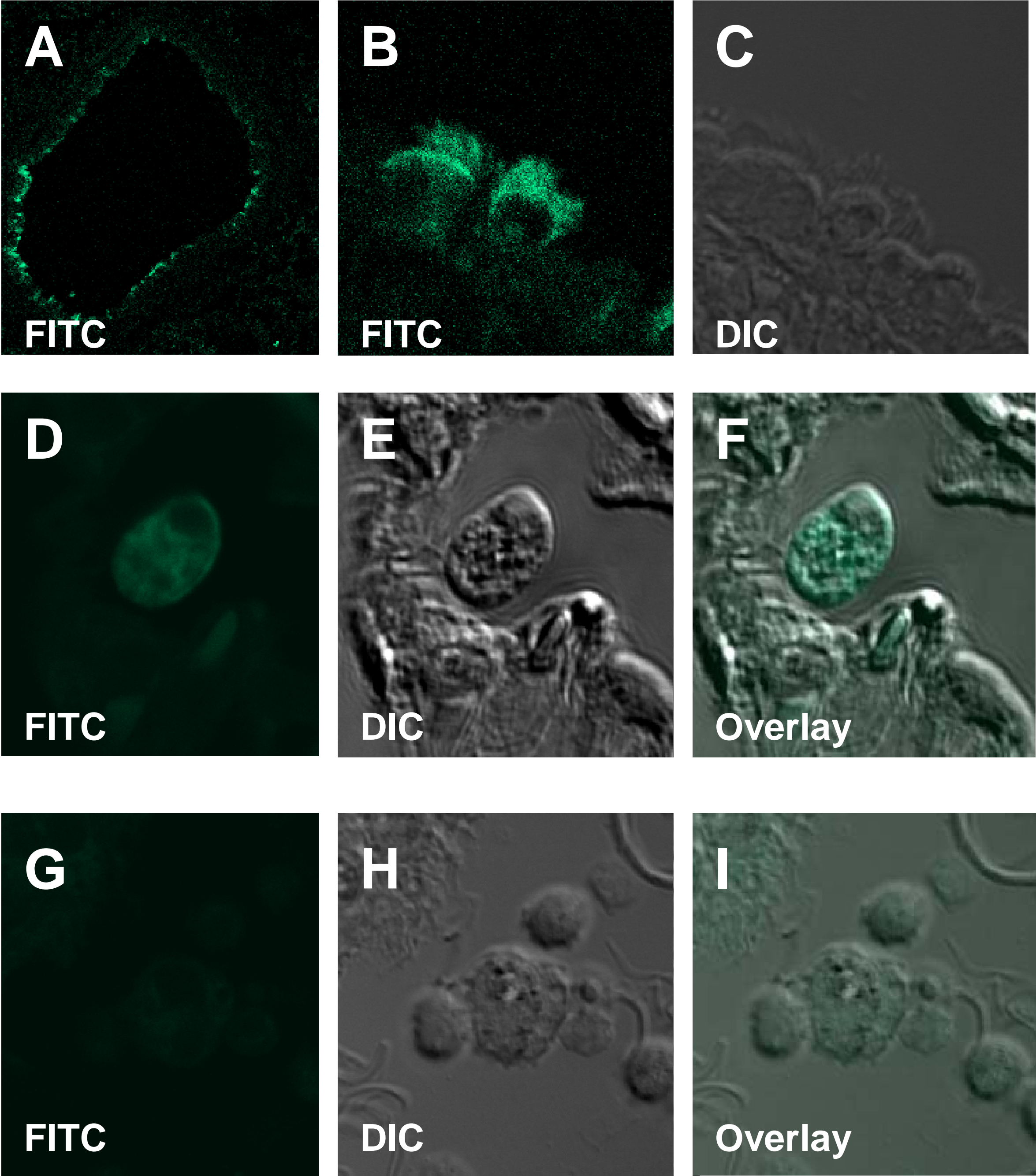
A.



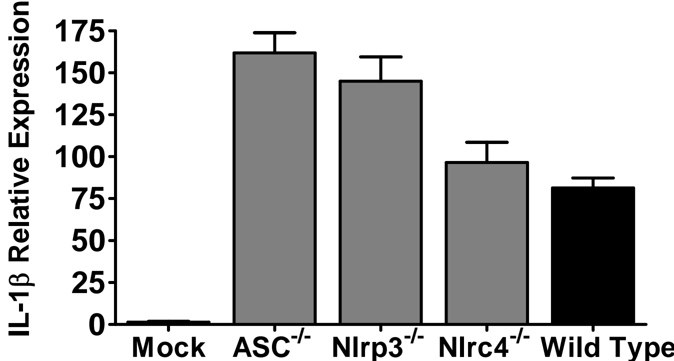
B.



Supplemental Figure S3.



Supplemental Figure S4.



Supplemental Figure S5.