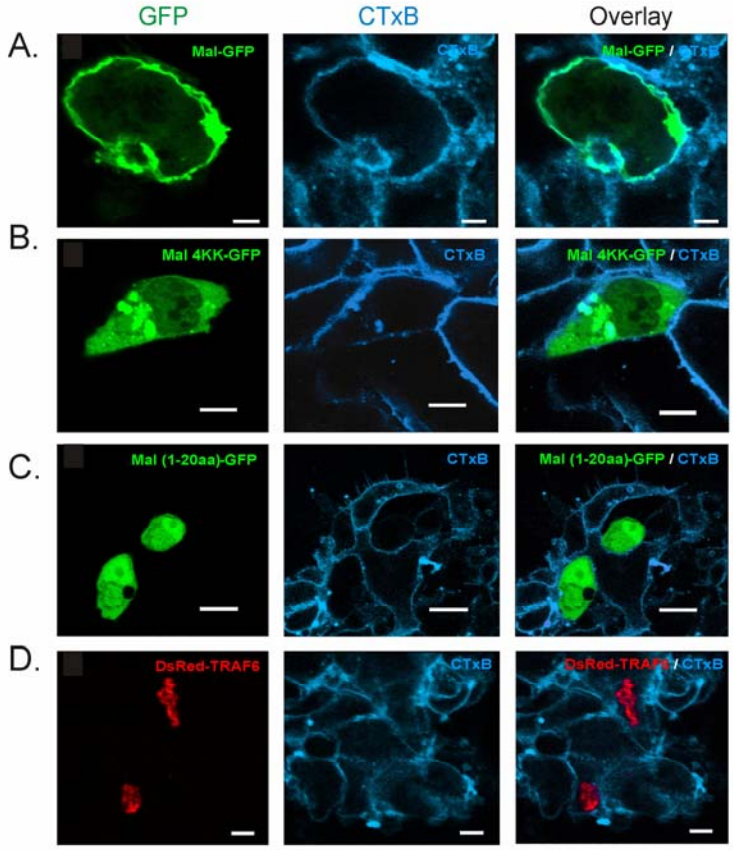
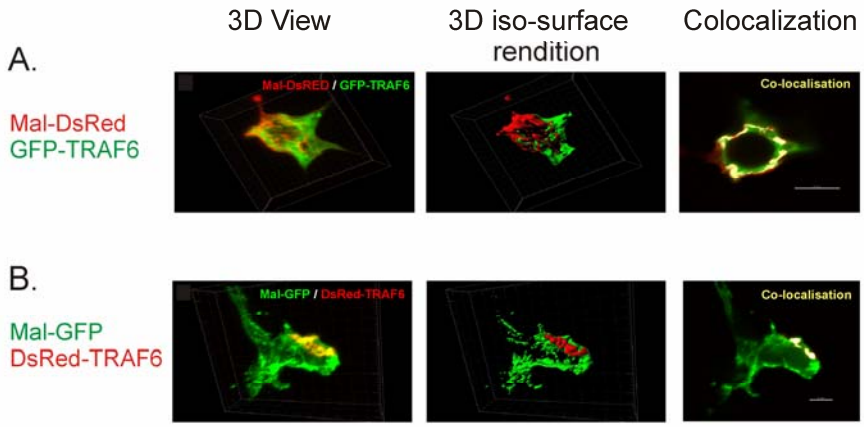
SUPPLEMENTARY DATA

Supplementary Data Figure 1



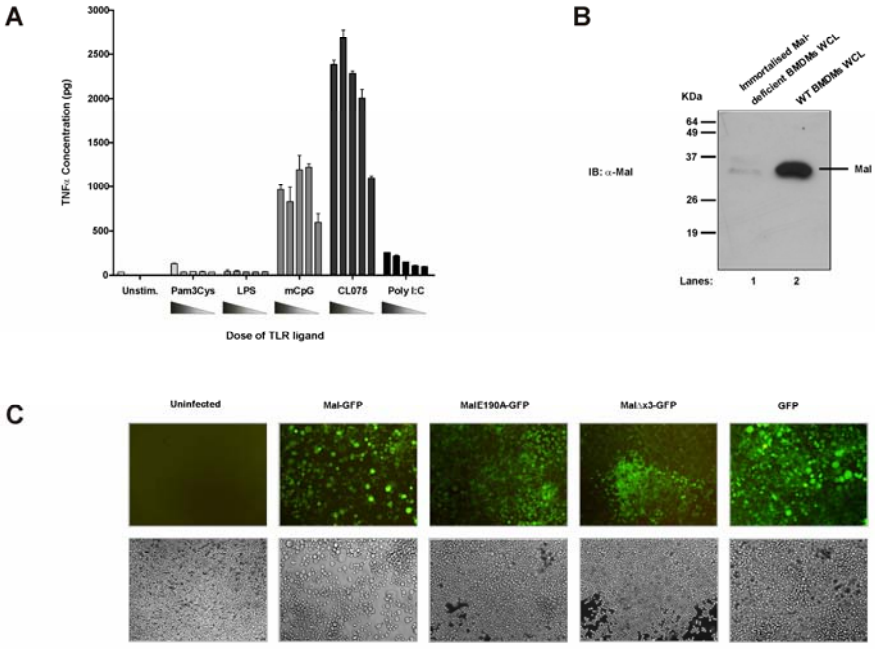
Supplementary Fig 1. Confocal imaging of Mal-GFP constructs and DsRed-TRAF6 in HEK293 cells. HEK293 cells were coated onto untreated glass coverslips at a cell density of 2 x 10^5 and co-transfected with 1.5 μg of plasmid DNA containing either (A) Mal-GFP , (B) Mal 4KK-GFP, (C) Mal (1-20aa)-GFP (0.3 μg DNA, respectively, Green) or (D) DsRed-TRAF6 (1.2 μg DNA, Red) for 48 h at 32ºC. The cells were fixed and counterstained with cholera toxin subunit B (CTxB) (Alexa Fluor 647) (Invitrogen) to visualise lipid rafts of the cell surface (blue, pseudocolor). (A) Mal-GFP shown in green is localised to the plasma membrane (bars 4 μm). (B-C) Mal 4KK-GFP and Mal (1-20aa)-GFP mutants are mis-localised and diffusely distributed throughout the cytoplasm (bars 8 μm and 16 μm, respectively). (D) DsRed-TRAF6 is found in discrete foci scattered throughout the cytosol (bars 8 μm). All images are single confocal sections taken with a 40X oil objective lens and are representative of at least three independent experiments where over 200 cells were examined per condition and, unless otherwise indicated in the text, >95% of the stained cells displayed similar staining.

Supplementary Figure 2



Supplemental Figure 2. Confocal co-localization imaging of Mal constructs with TRAF6 in HEK293 cells. HEK293 cells were coated onto untreated glass coverslips at a cell density of 2 x 10^5 and co-transfected with 1.5 μg of plasmid DNA containing either (A) Mal-GFP, (B) Mal-DsRed (0.3 μg, respectively) in conjunction with either DsRed-TRAF6 or GFP-TRAF6 (1.2 μg, respectively) for 48 h at 32ºC. Z-stack images were taken of individual cells and 3D image reconstruction was performed using Imaris software (BitPlane). (A) Co-localizationof the Z-stack images, in yellow, illustrates clusters of DsRed-TRAF6 co-localising with concentrated Mal-GFP in plasma membrane ruffles (right panel, bars 8 μm). (B) Co-localizationof the Z-stack images, in yellow, illustrates clearly GFP-TRAF6 co-localization with concentrated Mal-DsRed in plasma membrane ruffles (right panel, bars 8 μm). All images are single confocal sections taken with a 40X oil objective lens and are representative of at least three independent experiments where over 200 cells were examined per condition and, unless otherwise indicated in the text, >95% of the stained cells displayed similar staining.

Supplementary Figure 3



Supplementary Fig 3. Response and reconstitution of immortalised Mal-deficient macrophages. (A) Immortalised Mal-deficient macrophages were seeded at 2 x 10^5 cells/well and allowed to incubate overnight. Cells were treated with various TLR ligands in a dose-dependent manner for 24 hours: LPS (TLR4) (80, 40, 20, 10, 5 ng/ml), Pam3Cys (TLR2) (200, 100, 50, 25, 10 ng/ml), mCpG DNA (TLR9) (1000, 500, 250, 125, 62.5 μM), CL075 (TLR7) (2, 1, 0.5, 0.25, 0.125 μg/ml) and Poly I:C (TLR3) (500, 250, 125, 62.5, 31.25 μg/ml). Control unstimulated cells were included for comparison. Supernatants were collected and processed for TNFα concentrations (pg/ml) by ELISA. Data is presented as mean +/- SEM of duplicates within one experiment. (B) Mal-deficient (lane 1) or wild-type bone-marrow derived macrophages (lane 2) were seeded at 2 x 10^5 cells/well and incubated overnight. Whole cell lysates were harvested and detection of endogenous Mal was visualized by immunoblotting with anti-Mal antibody (Pearl-1, Dublin). Results are representative of two independent experiments. (C) Photomicrographs of fluorescent microscopy of Mal-deficient macrophages reconstituted with GFP-tagged genes expressing either: Mal-GFP, MalE190A-GFP, MalΔx3-GFP or GFP. The transduction efficiency of each macrophage population was determined to be 30-40% as assessed by FACS, of which GFP-positive populations for each gene were gated and sorted for seeding at a concentration of $3x10^5$ cells/well. Viable transduced GFP-positive immortalised Mal-deficient macrophages that had been cultured for 72 h following FACS were assessed by direct examination using fluorescence microscopy at an approximate confluency of 90% (top panels). Fluorescent images were taken with a 20X objective lens.