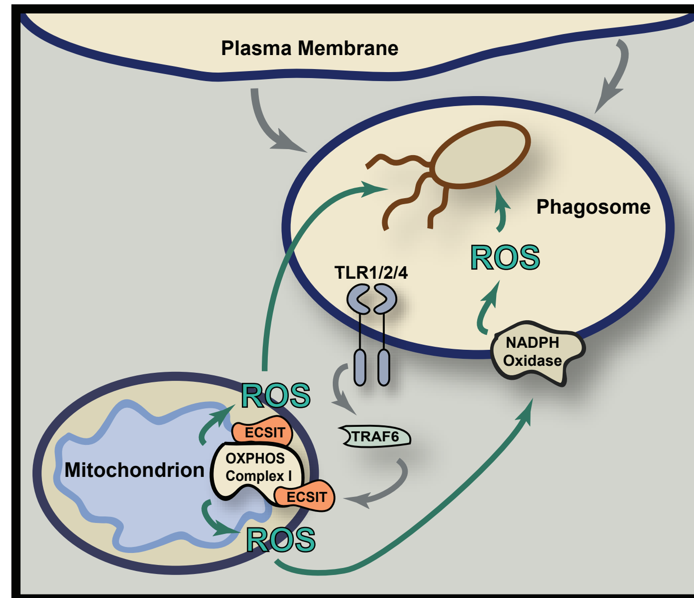
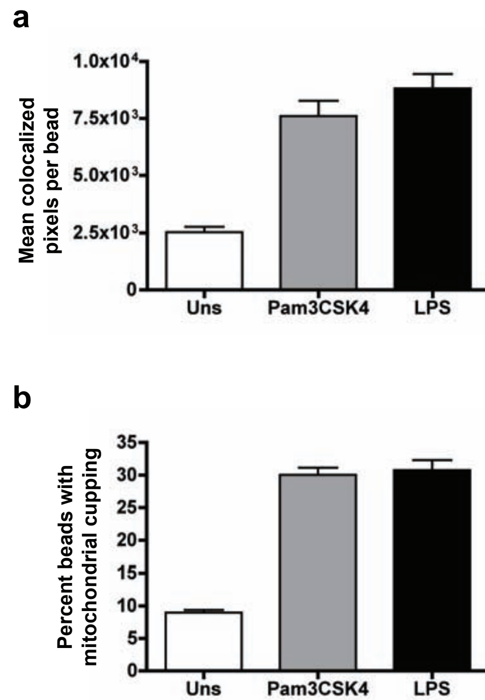
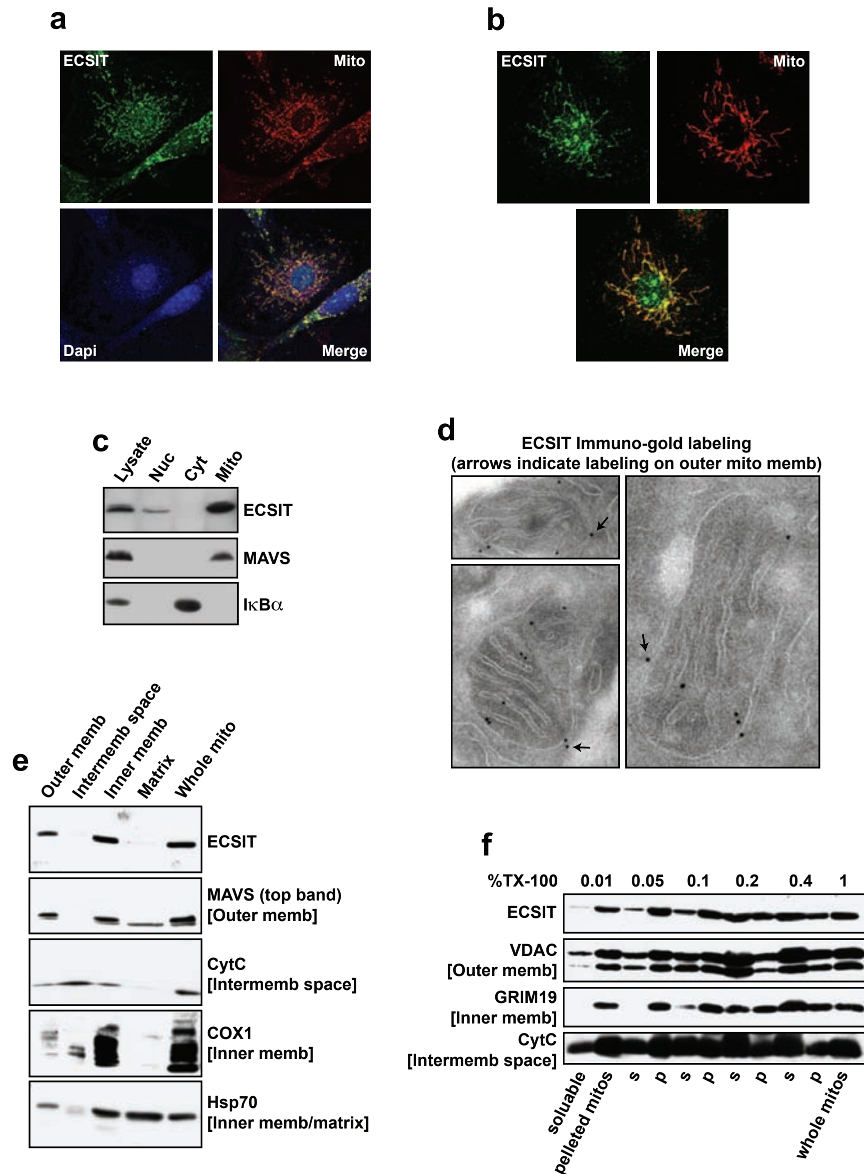


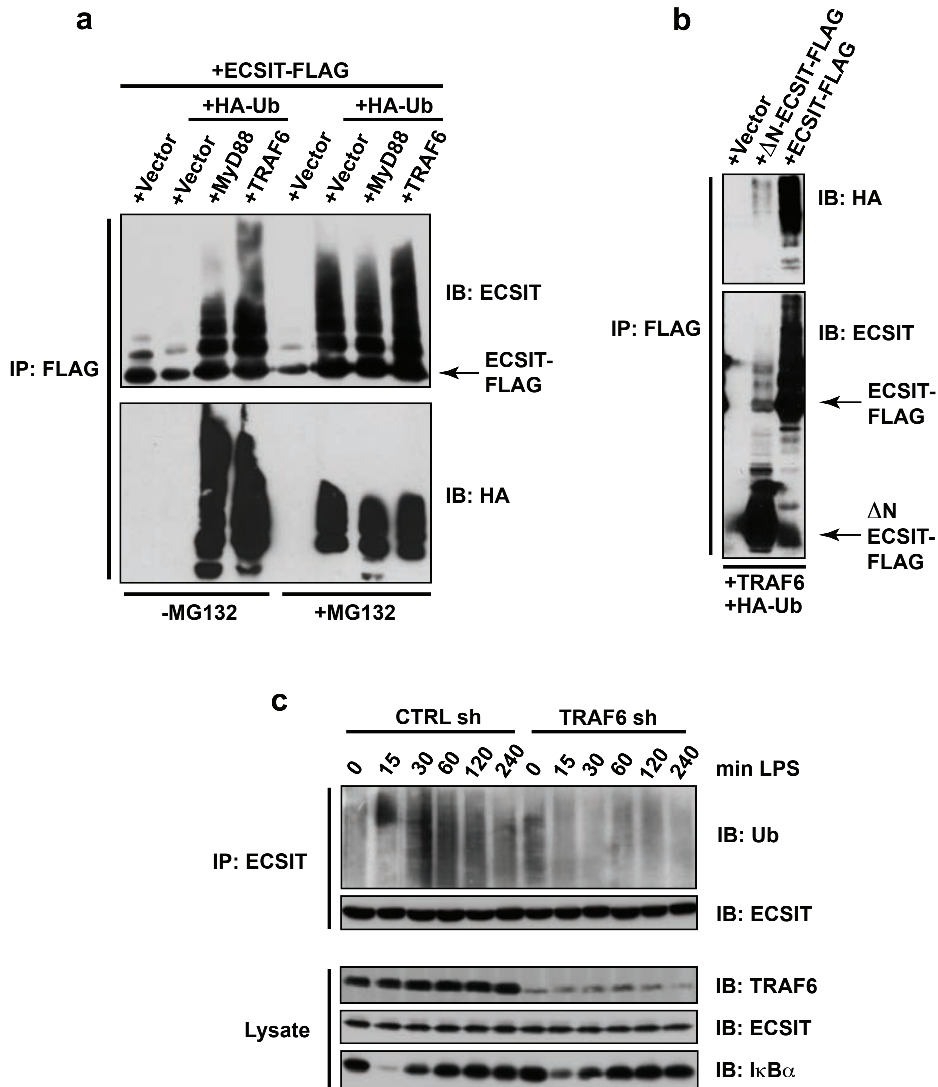

**Supplementary Figure 1 | Model detailing the roles of mitochondrial ROS in macrophage bacterial killing.** Agonists derived from phagocytized bacteria signal through TLR1/2, 2, or 4, leading to the recruitment of mitochondria to phagosomes and a concomitant trafficking of TRAF6 to peri-phagosomal mitochondria where it interacts with and ubiquitinates ECSIT. ECSIT ubiquitination and/or enrichment on the mitochondrial outer membrane alters OXPHOS activity, resulting in increased generation of the mROS superoxide. Superoxide is dismutated into hydrogen peroxide, which can freely cross mitochondrial membranes to augment ROS-dependent killing of bacteria. This can occur as mROS enters the phagosome and directly contributes to killing and/or by mROS priming of Phox signaling components for full ROS generation inside the phagosome.



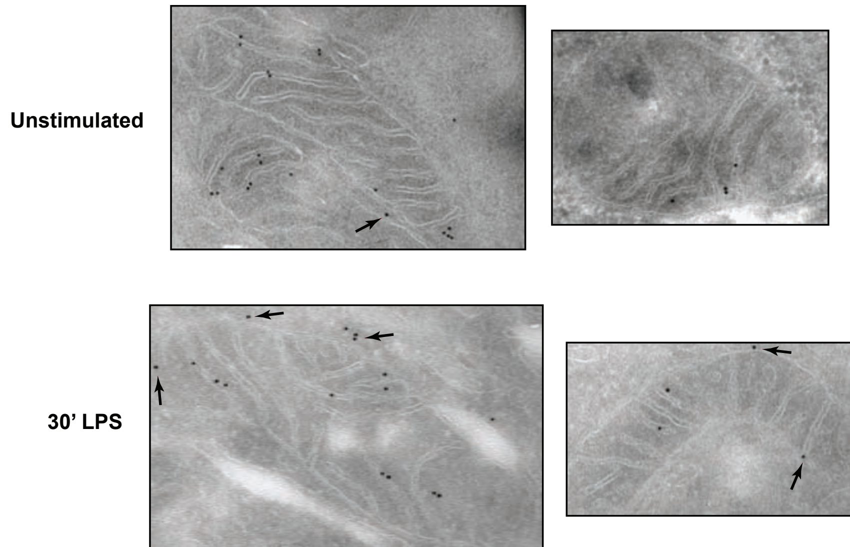

**Supplementary Figure 2 | TLR1/2/4 signaling induces mitochondrial recruitment and cupping around phagosomes. a-b**, BMM were incubated with either uncoated, Pam3CSK4, or LPS coated 3 micron Fluoresbrite latex beads, fixed, and mitochondrial networks were immunostained with HSP70 antibodies. Confocal Z-stacks were taken of three large fields of view, each containing approximately 30 cells, and colocalized images were generated. Mean colocalized pixels per bead (**a**) and percent beads with mitochondrial cupping (**b**) were calculated as described in Methods. Error bars indicate s.d. from the mean of triplicate images.

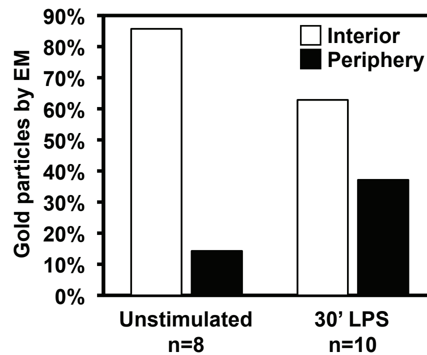



**Supplementary Figure 3 | ECSIT localizes to both mitochondrial membranes. a**, NOR10 fibroblasts were immunostained with antibodies against ECSIT or mitochondrial HSP60 [Mito]. Nuclei were stained with Dapi and images were collected and merged. **b**, BMM were immunostained with antibodies against ECSIT or HSP60 [Mito] and images were collected and merged. **c**, RAW cells were homogenized and subjected to differential centrifugation and sucrose gradient fractionation. Fractions were blotted with the indicated antibodies. **d**, J774 cells were stained with ECSIT antibody for immuno-electron microscopy analysis. Arrows indicate gold particles labeling the outer membrane. **e**, RAW cell mitochondria were subfractionated into outer membrane, intermembrane space, inner membrane, and matrix compartments or left intact and blotted as indicated. **f**, RAW mitochondria were subjected to increasing concentrations or 1% Triton X-100 to solubilize mitochondrial membranes or entire mitochondria, respectively. Samples were centrifuged to separate solubilized proteins [s] from remaining mitochondrial pellets [p] and blotted as indicated.

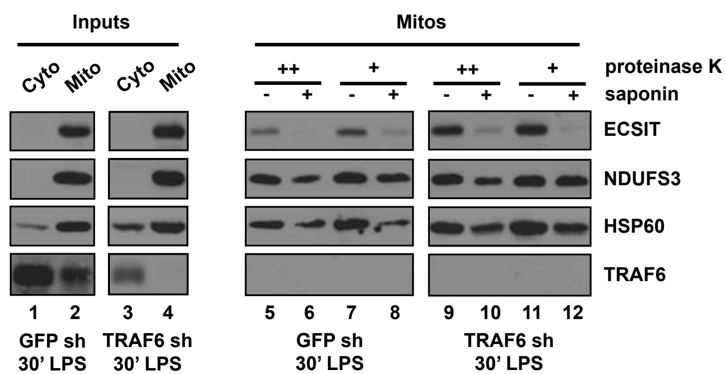



**Supplementary Figure 4 | ECSIT is ubiquitinated during TLR4 signaling in a TRAF6 dependent fashion. a-b**, 293T cells were transfected with the indicated constructs, incubated in MG132 or not, lysed in boiling SDS buffer, and immunoprecitated with FLAG antibody. Samples with either probed with ECSIT or HA antibodies to detect ubiquitin laddering. The ΔN-ECSIT-FLAG construct (**b**) lacks the N-terminal 260 residues required for TRAF6 interaction. **c**, RAW cells stably expressing CTRL or TRAF6 shRNA lentiviruses were stimulated with 1 μg/ml LPS for the indicated times and lysed with boiling SDS buffer to denature proteins and disrupt interactions. Samples were diluted in 2X TNT lysis buffer and immunoprecipitated with ECSIT antibody. Whole cell lysate and IP fractions were probed with the indicated antibodies.

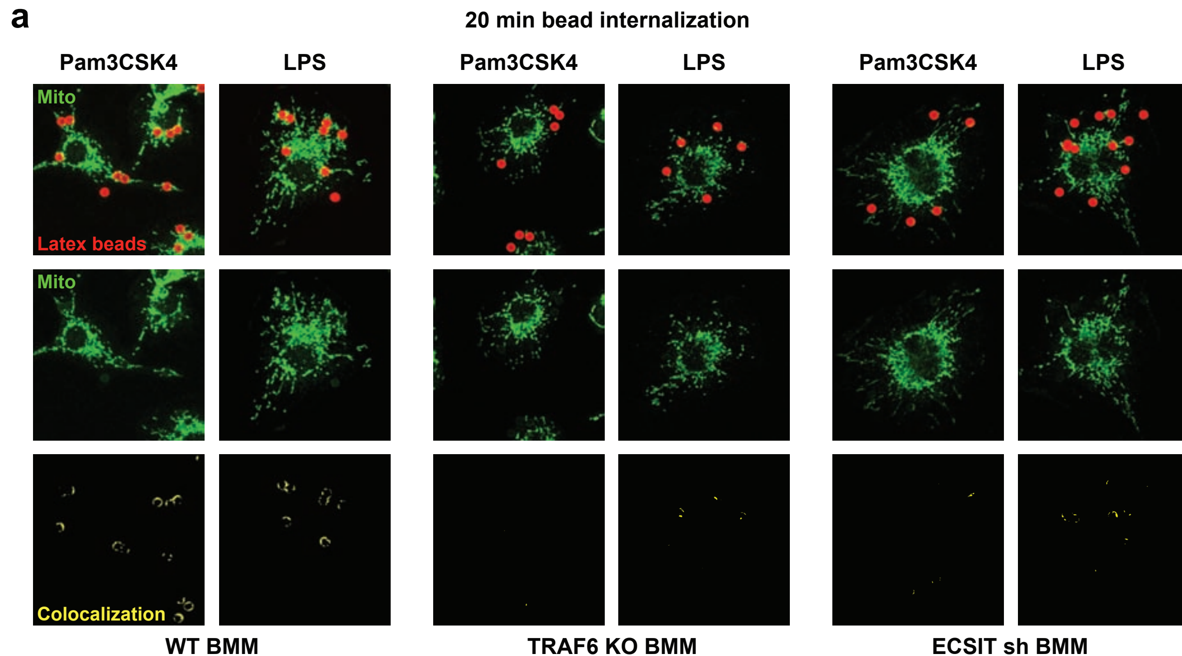


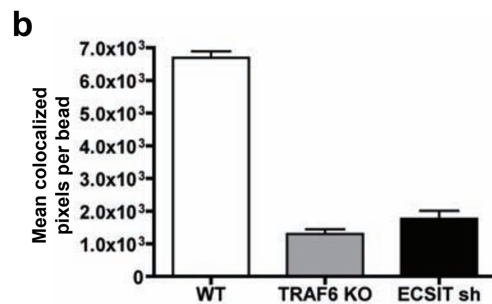




**Supplementary Figure 5 | ECSIT becomes more peripheral on mitochondria upon LPS stimulation.** J774 cells were stimulated as indicated, fixed, sectioned, and stained with ECSIT antibody for immuno-electron microscopy analysis. For graph at bottom, gold particles from EM images of mitochondria (number indicated below columns) were quantified and represented as percentage staining near inner (interior) or outer (periphery) membranes.

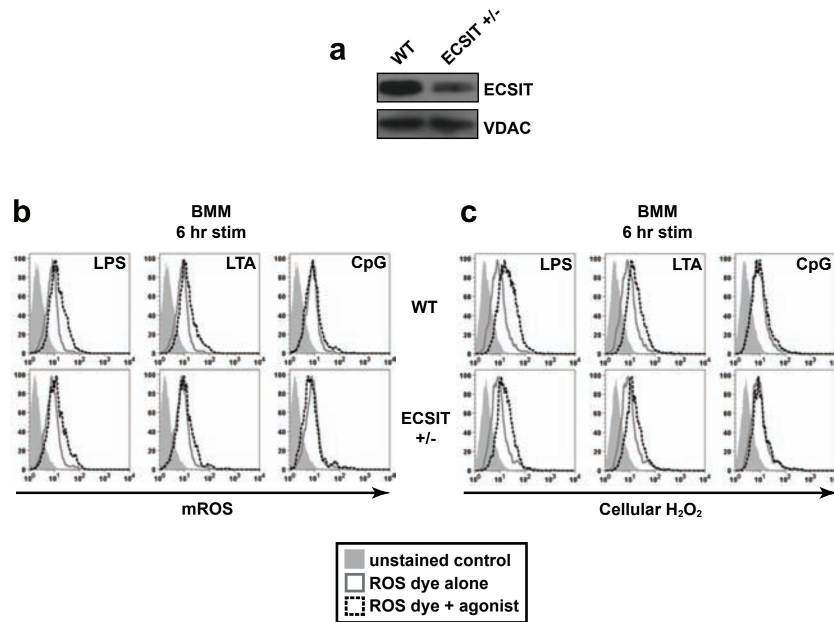



**Supplementary Figure 6 | TRAF6 is required for enrichment of ECSIT at the mitochondrial periphery upon TLR4 signaling, but does not enter mitochondria.** GFP sh or TRAF6 sh RAW cells were stimulated with 1 µg/ml LPS and mitochondria were fractionated. Equal amounts of extracts were treated with the indicated amount of proteinase K (++, 12 ng/µl; +, 6 ng/µl) on ice with or without 0.1% saponin to gently permeabilize mitochondrial membranes. Extracts were then blotted with the indicated antibodies.

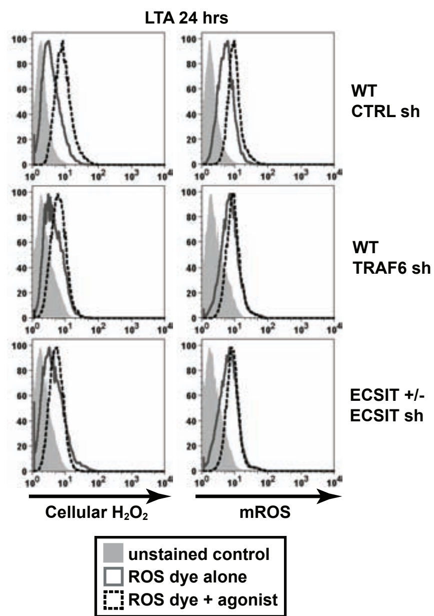


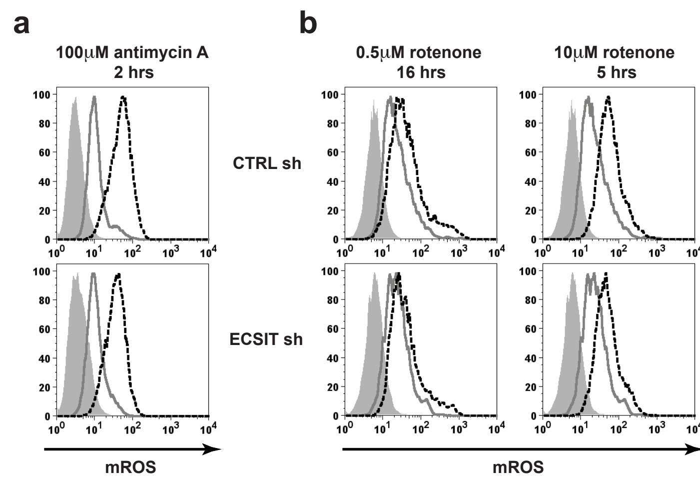

**Supplementary Figure 7 | TRAF6 and ECSIT are required for mitochondrial recruitment to phagosomes.** **a**, BMM were incubated with either Pam3CSK4 or LPS coated Fluoresbrite latex beads, fixed, and mitochondrial networks were immunostained with HSP70 antibodies [Mito]. Confocal Z-stacks were acquired and colocalized beads (red pixels) and mitochondria (green pixels) are displayed yellow (bottom). Images shown are representative of approximately 100 cells analyzed. **b**, Confocal Z-stacks were taken of three large fields of view from both Pam3CSK4 and LPS coated bead samples, each containing approximately 30 cells, and colocalized images were generated. Mean colocalized pixels per bead were calculated as described in Methods. Error bars indicate s.d. from the mean of triplicate images.



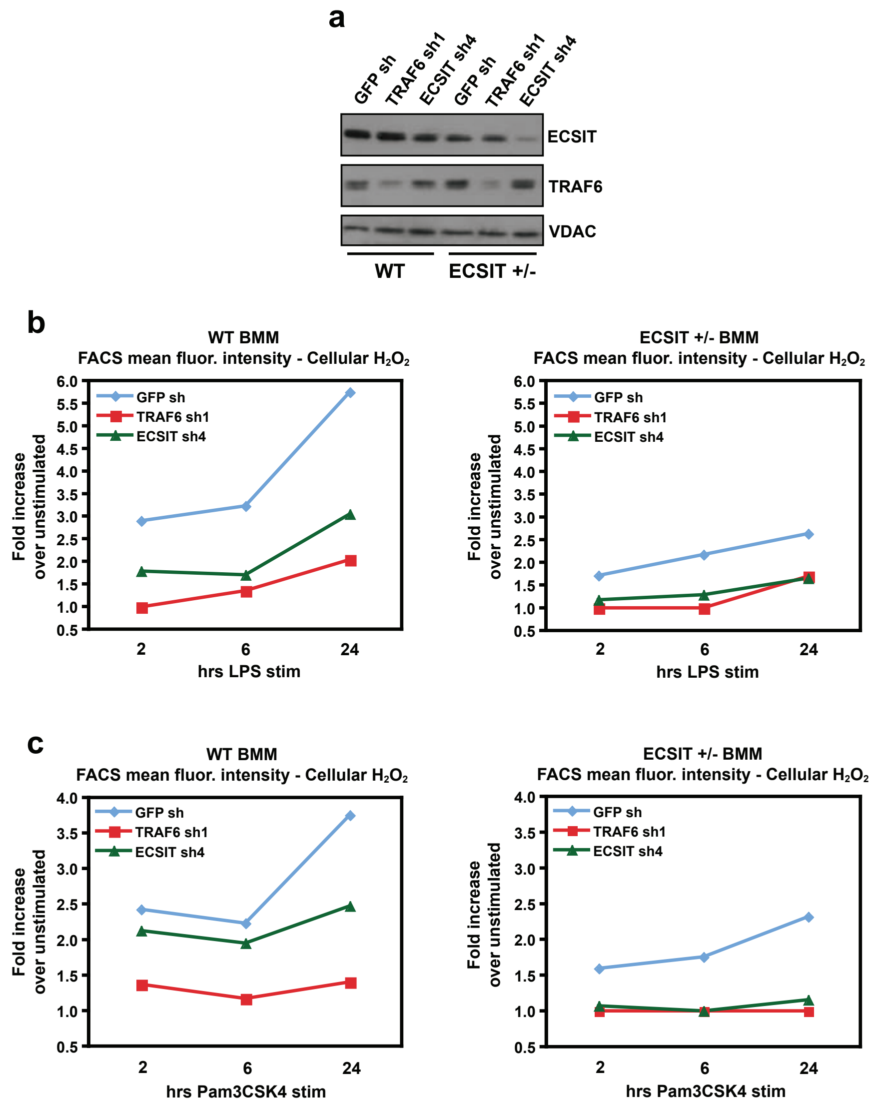

**Supplementary Figure 8 | ECSIT heterozygous BMM display decreased mitochondrial and cellular ROS upon TLR2/4 signaling. a**, WT or ECSIT +/- littermate BMM were solubilized with TNT lysis buffer and blotted using the indicated antibodies. **b-c**, WT or ECSIT +/- littermate BMM were left untreated or stimulated with 1 μg/ml LPS, 2 μg/ml LTA, or 1 μM CpG DNA for 6 hours. Cells were stained with MitoSOX [mROS] (**b**) or CM-$H_2$DCFDA [cellular $H_2O_2$] (**c**) and analyzed by FACS.



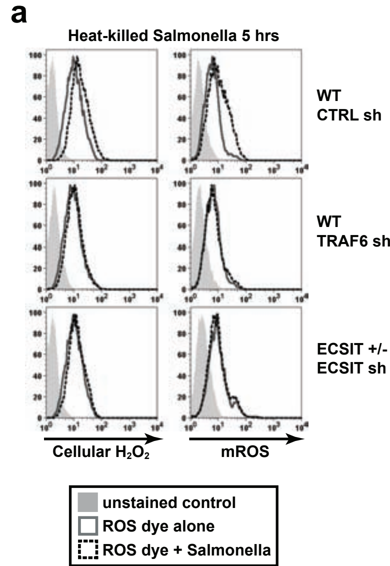

**Supplementary Figure 9 | TRAF6-ECSIT signaling controls ROS generation upon exposure to LTA.** WT or ECSIT +/- littermate BMM were infected with control, TRAF6, or ECSIT shRNA expressing lentiviruses and left untreated or stimulated with 2 µg/ml LTA for the indicated time. Cells were stained with MitoSOX and CM-$H_2$DCFDA and analyzed by FACS.

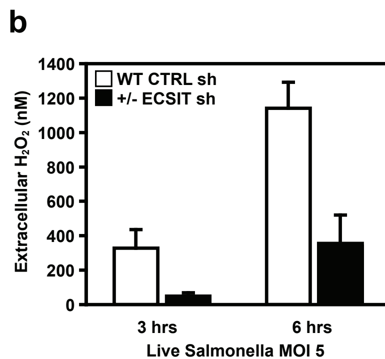



**Supplementary Figure 10 | ECSIT knockdown cells generate normal levels of mROS when exposed to antimycin A or rotenone. a-b**, RAW macrophages stably expressing CTRL or ECSIT shRNAs were treated with antimycin A (**a**) or rotenone (**b**) for the indicated times, stained with MitoSOX, and analyzed by FACS.

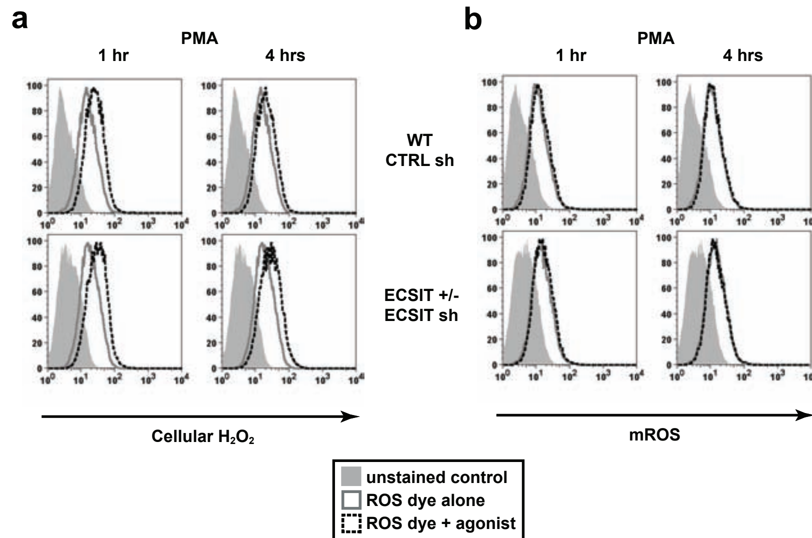


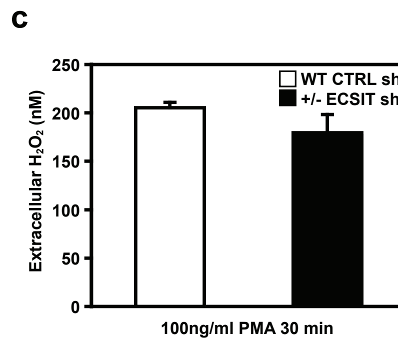

**Supplementary Figure 11 | TLR1/2/4 signaling induces cellular ROS through ECSIT and TRAF6.** **a**, WT or ECSIT +/- littermate BMM were infected with control GFP, TRAF6, or ECSIT shRNA expressing lentiviruses, solubilized in TNT lysis buffer, and blotted using the indicated antibodies. **b-c**, WT or ECSIT +/- littermate BMM were infected with GFP, TRAF6, or ECSIT shRNA expressing lentiviruses (unique shRNA sequences; see Methods for more information) and left untreated or stimulated with 500 ng/ml LPS (**b**) or 1 µg/ml Pam3CSK4 (**c**) for the indicated times. Cells were stained with CM-$H_2$DCFDA and analyzed by FACS. Mean fluorescence intensity fold changes were calculated as described in Methods.



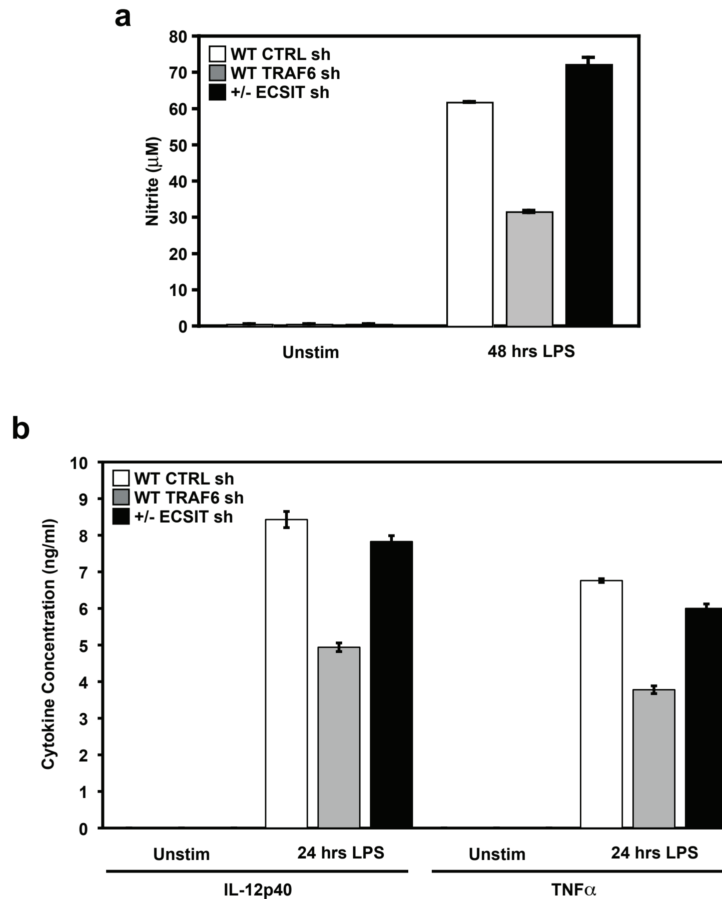




**Supplementary Figure 12 | TRAF6 and ECSIT deficient BMM produce less mROS and cellular $H_2O_2$ upon exposure to Salmonella. a**, WT or ECSIT +/- littermate BMM were infected with CTRL, TRAF6, or ECSIT shRNA expressing lentiviruses and left untreated or exposed to heat-killed Salmonella at a MOI of 50 bacteria/cell for 5 hours. Cells were stained with MitoSOX and CM-$H_2$DCFDA and analyzed by FACS. **b**, WT or ECSIT +/- littermate BMM were infected with CTRL or ECSIT shRNA expressing lentiviruses and left untreated or stimulated with live, serum opsonized Salmonella at a MOI of 5 bacteria/cell for the indicated times. Extracellular $H_2O_2$ production was measured by the Amplex Red Hydrogen Peroxide/Peroxidase assay. Error bars represent s.d. of the mean from triplicate samples.

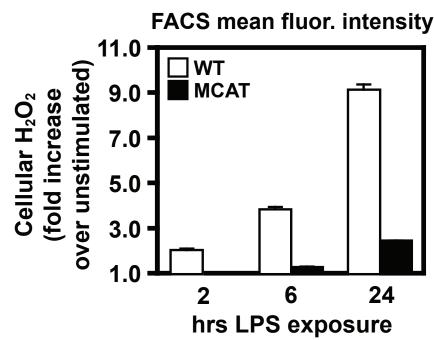



**Supplementary Figure 13 | ECSIT knockdown cells generate normal levels of ROS when exposed to PMA. a-c**, WT or ECSIT +/- littermate BMM were infected with CTRL or ECSIT shRNA expressing lentiviruses and left untreated or stimulated with 100 ng/ml PMA for the indicated times. Cells were then stained with CM-$H_2$DCFDA (**a**) or MitoSOX (**b**) and analyzed by FACS. Extracellular $H_2O_2$ production was measured by the Amplex Red Hydrogen Peroxide/Peroxidase assay (**c**). Error bars represent s.d. of the mean from triplicate samples.




**Supplementary Figure 14 | Nitric oxide and proinflammatory cytokine production are not inhibited in ECSIT deficient BMM. a-b**, WT or ECSIT +/- BMM were infected with CTRL, TRAF6, or ECSIT shRNA expressing lentiviruses and left untreated or stimulated with 500 ng/ml LPS for 48 hours. Supernatants were analyzed for nitrite generation by Griess reagent (**a**) or IL-12p40 and TNFα production by ELISA (**b**). Error bars represent s.d. of the mean from triplicate samples.




**Supplementary Figure 15 | MCAT macrophages generate less cellular ROS when stimulated with LPS.** WT and MCAT littermate BMM were left untreated or stimulated with 500 ng/ml LPS for 2, 6, or 24 hours. Cells were then stained with CM-$H_2$DCFDA and analyzed by FACS. Mean fluorescence intensity fold changes were calculated as described in Methods, and error bars represent s.d. of the mean from triplicate samples.

**Supplementary Table 1 │ Mitochondrial proteins identified in ECSIT tandem affinity purification from RAW 264.7 and HEK293 cells.**

| Entrez Gene ID | Description | RAW TAP Peptides | 293 TAP Peptides |
|---|---|---|---|
| 11740 | solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 5 | 5 | 9 |
| 15510 | heat shock protein 1, HSP60 | 5 | 10 |
| 229211 | acyl-Coenzyme A dehydrogenase family, member 9 | 12 | 9 |
| 298682 | ATPase family, AAA domain containing 3A | 1 | 6 |
| 498 | ATP synthase, mitochondrial F1 complex, alpha subunit, isoform 1 | 0 | 11 |
| 68349 | NADH dehydrogenase (ubiquinone) Fe-S protein 3 | 0 | 3 |
| 18674 | solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 3 | 0 | 3 |
| 69702 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor | 6 | 0 |