1 **Title**

Mitochondria-derived vesicles deliver antimicrobial payload to control phagosomal bacteria

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12 **Summary**

13

14 Pathogenic bacteria taken up into the macrophage phagosome are the target of many
15 anti-microbial effector molecules. Although mitochondria-derived antimicrobial effectors
16 such as reactive oxygen species (mROS) are reported to aid in bacterial killing, it is
17 unclear how these effectors reach bacteria within the phagosomal lumen. To examine
18 the crosstalk between mitochondria and phagosomes, we monitored the production and
19 the spatial localization of mROS during methicillin-resistant *Staphylococcus aureus*
20 (MRSA) infection. We showed here mROS, specifically hydrogen peroxide (mH_2O_2) can

be delivered into phagosomes via infection-induced mitochondria-derived vesicles, which are generated in a Parkin-dependent manner. Accumulation of mH_2O_2 in phagosomes required TLR signaling and the mitochondrial superoxide dismutase, Sod2, which converts superoxide into mH_2O_2. These data highlight a novel mechanism by which the mitochondrial redox capacity enhances macrophage antimicrobial function by delivering mitochondria-derived effector molecules into bacteria-containing phagosomes.

Introduction

Reactive oxygen species (ROS) play pivotal roles in signaling and defense of biological organisms. These highly reactive molecules oxidize lipids, proteins and other cellular constituents, leading to a spectrum of responses ranging from altered signaling to cell death. While ROS are constitutively generated and de-toxified during cellular metabolism, ROS levels acutely increase during cellular stress (Holmstrom and Finkel, 2014), and can be a potent weapon in the host arsenal to control invading pathogens. In cells of the innate immune system, like macrophages, ROS are primarily generated by the phagocyte NADH oxidase and mitochondrial metabolism. Upon infection, the phagocyte oxidase multi-protein complex, also referred to as NOX2, can be recruited to phagosomal membranes to generate a burst of superoxide into the phagosome lumen (Winterbourn and Kettle, 2013). However, many bacterial pathogens, like *Mycobacterium tuberculosis*, *Salmonella enterica* serovar Typhimurium, *Coxiella burnetti*, and *Francisella tularensis* prevent the rapid NOX2-mediated burst either by

avoiding complex recruitment or by utilizing detoxification mechanisms (Celli and Zahrt, 2013; Koster et al., 2017; Mertens and Samuel, 2012; Vazquez-Torres and Fang, 2001). Mitochondrial ROS (mROS) can also contribute to bacterial killing (West et al., 2011), but how these reactive molecules reach bacteria within the macrophage phagosome is ill-defined.

ROS induction can be driven by cellular stress response pathways (Bronner et al., 2015), like the endoplasmic reticulum unfolded protein response (UPR), which is activated by the three ER sensors, PERK, ATF6 and IRE1α (Zeeshan et al., 2016; Zhang and Kaufman, 2004). Indeed, many studies support an integral role for cellular stress pathways in modulating innate immune responses (Muralidharan and Mandrekar, 2013). We recently showed that IRE1α is critical for stimulating macrophage anti-microbial function (Abuaita et al., 2015). Specifically, IRE1α activation resulted in sustained macrophage ROS production required for killing MRSA. Notably, IRE1α dependent killing was only partially NOX2-dependent, despite the well established role of NOX2 in neutrophil oxidative defenses against *Staphylococcus aureus (Rigby and DeLeo, 2012)*. Since IRE1α signaling induces mROS production (Tufanli et al., 2017), we hypothesized that the mechanism by which IRE1α activation led to MRSA killing relied on mROS generation.

Here we show that infection of macrophages by MRSA stimulates IRE1α-dependent production of mROS, specifically hydrogen peroxide mH_2O_2. Infection also triggers generation of Parkin- dependent mitochondrial-derived vesicles (MDVs), previously

described as a pathway for mitochondrial quality control (Soubannier et al., 2012a). These MDVs deliver the mitochondrial peroxide-generating enzyme, Sod2, into the bacteria-containing phagosome, controlling bacterial burden. Our findings reveal a mechanism by which programmed cellular stress responses repurpose a mitochondrial quality control mechanism to enable anti-microbial defense.

Results

Induction of mROS by IRE1α promotes macrophage bactericidal function

We previously showed that MRSA infection stimulates macrophages to produce global ROS via the activation of the ER stress sensor IRE1α, which was required *in vitro* and *in vivo* for effective MRSA killing (Abuaita et al., 2015). Although the phagocyte NADPH oxidase-2 (NOX2) complex contributed somewhat to MRSA killing by macrophages, IRE1α stimulated robust macrophage bactericidal activity even in the absence of NOX2. Previous reports indicated that mitochondrial ROS (mROS) enhance macrophage bactericidal function (Geng et al., 2015; West et al., 2011), so we hypothesized that IRE1α-dependent antimicrobial activity might rely specifically on mROS. To test this hypothesis, we assessed whether mROS was induced by macrophages in response to MRSA infection. First, we validated that MitoPY1, a mitochondrially targeted probe that fluoresces in response to mH_2O_2 (Dickinson and Chang, 2008; Dickinson et al., 2013), was indeed mitochondrially restricted in macrophages. RAW264.7 cells were transfected with mito-mCherry and loaded with MitoPY1. MitoPY1 fluorescence intensity increased when macrophages were stimulated with exogenous H_2O_2, and the signal co-

90 localized with mito-mCherry (Fig. 1A). We also quantified the difference in mean
91 fluorescence intensity (MFI) by flow cytometry (Fig. 1B). During MRSA infection,
92 MitoPY1 fluorescence intensity increased over time, peaking at 4h pi (Fig. 1C). To
93 determine whether IRE1α was required for mH_2O_2 induction by MRSA infection, we
94 generated IRE1α-deficient macrophages using CRISPR/Cas9 (Fig. 1D and S1). IRE1α
95 deficiency suppressed the ability of macrophages to induce mH_2O_2 during MRSA
96 infection when compared to non-target control (Fig. 1E). To test the requirement for
97 mROS in killing MRSA, infected macrophages were treated with the ROS scavenger,
98 NecroX-5, which is primarily localized to mitochondria (Kim et al., 2010; Thu et al.,
99 2016). Infected macrophages treated with NecroX-5 exhibited lower MitoPY1
100 fluorescence than control-treated cells (Fig. 1F), and decreased capacity to kill MRSA
101 (Fig. 1G). These data indicate that IRE1α is critical for infection-induced mH_2O_2, and
102 establishes a role for mROS in macrophage bactericidal function against MRSA.
103
104 **Mitochondrial peroxide accumulation in phagosomes is TLR-dependent**
105 We reasoned that mH_2O_2 could contribute to bactericidal function indirectly by signaling
106 and/or by direct delivery to the phagosome. If direct delivery, we might expect to see
107 mH_2O_2 accumulate in the phagosome. To monitor mH_2O_2 spatial localization during
108 infection, we imaged live cells stimulated with viable MRSA, killed MRSA or latex beads.
109 Macrophages were pulsed with MitoPY1 and chased 4h post-phagocytosis (Fig. 2A).
110 Hydrogen peroxide increased within the mitochondrial network during infection with live
111 or fixed MRSA, but not with beads. We also observed smaller MitoPy1^{+} puncta
112 throughout the cell. Notably, mH_2O_2 accumulated in MRSA-containing phagosomes

(Fig. 2A and 2B). To visualize the dynamic distribution of mH_2O_2 during infection, we performed time-lapse imaging of infected macrophages pre-loaded with MitoPY1 (Movie S1). By 10 min pi, the MitoPY1 signal within the mitochondrial network had increased. MitoPy1$^+$ puncta first associated with the bacterial phagosome at approximately 50 min pi, followed by accumulation of probe within the bacteria-containing phagosome (Fig. 2C and Movie S1). These data suggest that mitochondrially-derived hydrogen peroxide accumulates within phagosomes, and reveal the possibility that mH_2O_2 may contribute to macrophage bactericidal effector function through a direct delivery mechanism.

Mitochondrial ROS induction in macrophages occurs when cells internalize beads conjugated to Toll-like receptor (TLR) ligands (TLR2 and TLR4), but not beads alone (West et al., 2011). To test whether TLR signaling was required for mH_2O_2 generation and accumulation within MRSA-containing phagosomes, we measured MitoPY1 fluorescence intensity in wild-type (WT) and TLR2/4/9-deficient bone marrow-derived macrophages (BMDMs) during MRSA infection. We observed increased overall mH_2O_2 in both WT and TLR2/4/9 deficient BMDMs during MRSA infection, indicating that mH_2O_2 induction is largely independent of TLR2/4/9 signaling (Fig. S2A). However, TLR2/4/9-deficient macrophages failed to kill MRSA (Fig. S2B) and MitoPY1 accumulation in their MRSA-containing phagosomes was decreased compared to WT macrophages (Fig. 2D and 2E), indicating that TLR signaling controls mH_2O_2 delivery to or accumulation within bacteria-containing phagosomes.

Bacterial infection triggers Parkin-dependent generation of mitochondrial-derived vesicles

Recent studies have revealed that mitochondrial constituents can be selectively transported to other intracellular compartments by small mitochondrial-derived vesicles (MDVs) (McLelland et al., 2014; Soubannier et al., 2012a). MDVs form in an early dynamin related protein 1 (Drp1)-independent response to increased oxidative stress, and are positive for mitochondrial markers, like Tom20. MDVs may function as a quality control mechanism by delivering damaged mitochondrial components to the endolysosomal pathway (Sugiura et al., 2014). A subset of MDVs associate with peroxisomes, and may represent a mechanism of communication between these two organelles (Neuspiel et al., 2008). Since infection increases mH_2O_2, we reasoned that could trigger generation of MDVs, a potential mechanism to deliver antimicrobial content from the mitochondria to the phagosome. To test this hypothesis, we first assessed whether MDVs are induced by MRSA infection. Macrophages were infected with MRSA, and subjected to immunofluorescence analysis by high-resolution confocal microscopy using a Tom20-specific antibody. MRSA infection stimulated an increase of small $Tom20^+$ particles, here referred to as MDVs, compared to bead-containing macrophages (Fig. 3A and 3B, Fig. S3). The Parkinson's Disease associated protein, Parkin, regulates biogenesis of a subset of MDVs (McLelland et al., 2014). We therefore tested the requirement for Parkin in generating infection-induced MDVs. WT and Parkin-deficient (*Park2*$^{-/-}$) BMDM were infected with MRSA, and accumulation of $Tom20^+$ MDVs visualized by confocal microscopy. MRSA-infected *Park2*$^{-/-}$ BMDM had significantly lower numbers of $Tom20^+$ MDVs compared to WT BMDM (Fig. 3C and 3D).

158 Collectively, these results show that MRSA infection induces formation of $Tom20^{+}$
159 MDVs through a Parkin-dependent mechanism.
160
161 **Parkin controls mH_2O_2 accumulation in phagosomes and promotes bactericidal**
162 **effector function**
163 Polymorphic alleles of *Park2* can mediate susceptibility to microbial infection (Al-
164 Qahtani et al., 2016; Manzanillo et al., 2013). We hypothesized that MDVs could
165 enhance macrophage bactericidal function by facilitating mH_2O_2 accumulation within
166 phagosomes. We first measured the requirement for Parkin in mH_2O_2 accumulation in
167 phagosomes. Upon infection, Parkin-deficient macrophages produced higher levels of
168 mH_2O_2 than WT macrophages, indicating that Parkin is not necessary for mH_2O_2
169 induction (Fig. 3E). Notably, despite higher overall levels of mH_2O_2, Parkin-deficient
170 macrophages displayed only minimal accumulation of MitoPY1 in phagosomes
171 compared to WT macrophages (Fig. 3F and 3G). To evaluate the contribution of Parkin
172 to macrophage bactericidal capacity against MRSA, WT and *Park2*$^{-/-}$ BMDM were
173 infected with MRSA to assess killing. Parkin-deficient macrophages were less capable
174 of killing MRSA compared to WT macrophages (Fig. 3H). Parkin can regulate
175 mitochondria quality control by inducing mitophagy (Matsuda et al., 2010), or by
176 formation of MDVs (McLelland et al., 2014). To determine if the Parkin-dependent killing
177 mechanism we observed involved mitophagy, we measured macrophage bactericidal
178 function in the presence of Mdivi-1, a small molecule of Drp1, required for mitophagy
179 (Narendra et al., 2008), but not MDV scission (Soubannier et al., 2012a). Macrophages
180 treated with Mdivi-1 killed MRSA as efficiently as DMSO-treated macrophages (Fig. 3I).

These data indicate that Parkin-dependent generation of MDV enables accumulation of bactericidal mH_2O_2 in the macrophage phagosome.

We then tested the role of Parkin in innate immunity against MRSA using a subcutaneous infection model, where innate immune defenses are essential for bacterial clearance (Tseng et al., 2011). WT and Parkin-deficient mice were inoculated with 10^7 MRSA subcutaneously into the shaved flank. Skin lesions were excised at 3 days pi, and bacterial burden and pro-inflammatory cytokines were measured. Lesions from Parkin-deficient mice yielded higher bacterial numbers (Fig. 4A), and increased levels of KC and IL-1β compared to WT mice (Fig. 4B and 4C). These data support a role for Parkin in innate immunity against MRSA infection *in vivo* and suggest that parkin mediates macrophage bactericidal function via delivering $Tom20^+$ MDV into phagosomes.

Inhibition of phagosomal acidification enhances MDV-mediated bactericidal capacity

Phagosomal acidification enhances macrophage antimicrobial function by reducing intracellular replication of certain pathogens such as *M. tuberculosis* (Sullivan et al., 2012). Conversely, other intracellular bacterial pathogens, such as *Salmonella enterica* serovar Typhimurium, are killed more efficiently when cells are treated with Bafilomycin A1 (Baf A1), an inhibitor of the vacuolar ATPase that prevents acidification and phagolysosomal fusion (Rathman et al., 1996). Notably, under stimulating conditions, $Tom20^+$ MDVs accumulate when cells are treated with BafA1, presumably by

preventing their resolution into the lysosomal network and subsequent degradation (Soubannier et al., 2012a; Sugiura et al., 2014). Therefore, we hypothesized that blockade of acidification and protein degradation in lysosomes with Baf A1 (Yoshimori et al., 1991) would increase MDV accumulation and thereby enhance bactericidal mROS delivery into MRSA-containing phagosomes. To determine if MDV accumulation enhanced macrophage bactericidal function, we first quantified MDV induction in infected macrophages treated with BafA1. We observed that BafA1 treatment increased Tom20^{+} MDV accumulation in MRSA-infected macrophages compared to DMSO-treated macrophages (Fig. 5A and 5B). BafA1-treated macrophages exhibited higher levels of mROS in MRSA-containing phagosomes and killed MRSA more efficiently compared to DMSO-treated cells (Fig. 5C-E). To further investigate how MDVs associate with MRSA-containing phagosomes, we performed transmission electron microscopy (TEM) on MRSA-infected macrophages treated with or without BafA1 at 4h pi. We could readily observe MRSA-containing phagosomes containing double membrane-bound vesicles (Fig. 5F). When cells were treated with BafA1, MRSA phagosomes appeared more spacious and the double membrane-bound vesicles could be easily observed inside the phagosome. To determine if these vesicles were derived from mitochondria, we performed immunogold labeling using anti-Tom20 antibody, followed by TEM. Although the immunogold fixation conditions decreased definition of phagosomal membranes, Tom20^{+} particles were observed in close proximity to the bacterial surface within the phagosomal space (Fig. S4). Collectively, these data suggest that preventing lysosomal acidification and protein degradation increased MDV accumulation and mH_2O_2 levels in the phagosome to augment MRSA killing.

Sod2 is required for generation of bactericidal mH_2O_2 and is delivered to bacteria-containing phagosomes

Hydrogen peroxide is constitutively generated in the mitochondria from superoxide produced by electron transport that is converted by the mitochondrial manganese superoxide dismutase (Sod2) (Murphy, 2009). We therefore hypothesized that Sod2 was required for the bactericidal mH_2O_2 induced by MRSA infection. To define the spatial localization of Sod2 positive compartments relative to phagosomes, we performed confocal immunofluorescence microscopy on macrophages infected with MRSA or beads, staining with Sod2- and Lamp1-specific antibodies (Fig. 6A). Sod2 localized to the mitochondrial network in macrophages (Fig. S5) and large $Sod2^+$ network objects were juxtaposed to both bead- and MRSA-containing phagosomes. However, small $Sod2^+$ vesicles were induced during MRSA infection, and were present within MRSA-containing phagosomes. To further quantify $Sod2^+$ vesicle accumulation in MRSA-containing phagosomes, we enumerated $Sod2^+$ MDV located within a 1μm radius around bead- and MRSA-containing phagosomes, which were delineated by $Lamp1^+$ staining, and found that MRSA infection increased $Sod2^+$ MDV within phagosomes (Fig. 6B and 6C). To determine whether Sod2 was required for bactericidal activity, we stably knocked down Sod2 in RAW264.7 macrophages (Sod2 KD), which was confirmed by immunoblot analysis (Fig. 6D). We first tested the requirement of Sod2 in hydrogen peroxide and superoxide generation during MRSA infection (Fig. 6E, S6A and S6B). Compared to control cells, Sod2 KD cells failed to produce hydrogen peroxide upon MRSA infection while produced higher level of

250 superoxide regardless of MRSA infection. Although Sod2 knockdown increased
251 mitochondria superoxide production, it did not interfere with host cell death during
252 MRSA infection (Fig. S6C). Importantly, Sod2 depletion impaired macrophage killing of
253 MRSA compared to NT-control cells (Fig. 6F). Together, these results suggest that
254 Sod2 enhances MRSA killing via generation of mH_2O_2, which is delivered by MDVs to
255 bacteria-containing phagosomes.
256
257 **DISCUSSION**
258
259 The generation of anti-microbial reactive oxygen intermediates is an integral weapon in
260 the innate immune arsenal. We find that infection by MRSA stimulates the production of
261 mitochondrial ROS, specifically mitochondrial hydrogen peroxide. Our results identify
262 Sod2 as a key enzyme responsible for infection-induced mH_2O_2 generation. Moreover,
263 these studies reveal delivery of the Sod2/mH_2O_2 payload to the phagosome by
264 mitochondria-derived vesicles as a novel mechanism for anti-microbial killing.
265 Stimulation of mH_2O_2-containing MDV during infection required TLR signaling and
266 employed a Parkin-dependent pathway. Taken together, our data support a model
267 where Sod2-driven mH_2O_2 production and accumulation via MDV delivery establish a
268 potent killing ground for bacterial pathogens within the macrophage phagosome.
269
270 Generation of MDV has been described as a quality control mechanism that could
271 transport damaged respiratory chain complexes from mitochondria to the
272 endolysosomal compartment for degradation (Soubannier et al., 2012a). More recent

studies reveal that MDV can carry out diverse functions within the cell, and likely represent multiple vesicle populations with distinct cargo related to their function. Sugira, *et al*, provided evidence for MDV generation as a critical step in peroxisome biogenesis, where MDV containing the peroxisomal proteins, Pex3 and Pex14, fuse with ER-derived vesicles, containing Pex16, resulting in import-competent organelles (Sugiura et al., 2017). This model of peroxisome biogenesis adds a new dimension to our understanding of how MDVs carry out mitochondrial communication with other organelles. Our work further identifies the phagosome as a new destination for MDVs that is revealed by infection.

Soubannier *et al* showed that different proteins are incorporated in MDVs when mitochondria are stressed with different stimuli, implying the existence of mechanisms to package and transport specific cargos. For instance, exogenous ROS applied to isolated mitochondria results in MDVs enriched in the outer membrane protein, VDAC. However, when ROS is generated by isolated mitochondria via inhibition of complex III by Antimycin A, MDVs carried complex III subunit core2 without enrichment of VDAC (Soubannier et al., 2012b). A recent study showed that in response to heat shock or LPS, MDVs delivered mitochondrial antigens to antigen-loading compartment independently of Parkin (Matheoud et al., 2016). In contrast, when cells are treated with Antimycin A to stimulate mitochondrial ROS production, MDVs are targeted to lysosomes in a Parkin-dependent manner (McLelland et al., 2014). In the context of infection, our data demonstrated that MDVs are generated in a Parkin-dependent manner, similar to MDVs destined for lysosomes. Moreover, we showed that infection-

induced MDVs contain mitochondria-specific enzyme, Sod2. Collectively, our data and others support the idea that specific stimuli define the packaging and destination of MDV subsets.

In the context of the mitochondrial matrix, Sod2 cooperates with other enzymes and anti-oxidant proteins to detoxify superoxide generated by oxidative metabolism. Notably, dysregulation of Sod2 is associated with many human diseases, some of which are associated with increased inflammation (Flynn and Melov, 2013). Altering the spatial distribution of Sod2 could disrupt the coordinated ROS detoxification process, resulting in increased hydrogen peroxide production without the capacity to further reduce to water. Previous studies provide some evidence to support this hypothesis. Overexpression of Sod2 increased the steady state of hydrogen peroxide in cancer cells, and may contribute to tumor invasion and metastasis (Nelson et al., 2003; Ranganathan et al., 2001). In contrast, decreasing Sod2 activity in Sod2-heterozygous mice led to higher production of superoxide radical when measured by aconitase activity, which correlate with an increase in mitochondrial oxidative damage (Williams et al., 1998). Consistent with these data, our results showed that decreasing macrophage Sod2 leads to elevation of steady state levels of mitochondrial superoxide and decreased generation of hydrogen peroxide during MRSA infection. As a consequence, Sod2-deficient macrophages (Sod2 KD) failed to kill MRSA. Although we were not able to successfully generate live Sod2-deficient mice, studies of Sod2 knockdown in zebrafish demonstrate a protective role for Sod2 during infection by *Pseudomonas aeruginosa* (Peterman et al., 2015). Our studies have identified MDVs as a new delivery

319 mechanism by which antimicrobial effectors can be trafficked into the macrophage
320 phagosome. We propose that removal of a subset of Sod2 protein from the coordinated
321 redox environment of the mitochondrial network through packaging into MDVs allows
322 repurposing of this enzyme from detoxification to anti-microbial defense.

323

324 **Acknowledgments**

325 This work was supported by NIH award R21 AI101777 (M.X.O). B.H.A. was supported
326 by NIH T32 AI007538, T32 HLL007517 and an American Heart Association Post-
327 doctoral fellowship. We thank O'Riordan lab members for many helpful discussions. We
328 gratefully acknowledge the Center for Live Cell Imaging (CLCI), Microscopy and Image
329 Analysis Laboratory (MIL) and Cancer Center Immunology Core at the University of
330 Michigan Medical School. We thank Dr. T. Merkel (FDA) for *Tlr*2/4/9$^{-/-}$ femurs.

331

332 **Author Contributions**

333 B.A. performed the experiments; B.A. and M.O. designed the experiments and wrote
334 the manuscript; T.S. assisted in experimental preparation.

335

336 **Declaration of Interests**

337 The authors declare no competing interests.

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Figure Legends

Figure 1. MRSA infection stimulates bactericidal mH_2O_2 via IRE1α

(A) Representative live fluorescent images of macrophages transfected with mCherry-Mito-7 encoded plasmid, pulsed with MitoPY1 for 1h and chased with H_2O_2 (100 μM) or left untreated. Images were acquired by Olympus IX-70 inverted microscope and analyzed by MetaMorph for Olympus imaging software.

(B) Flow Cytometry mean fluorescent intensity (MFI) of macrophages after pulse with MitoPY1 for 1h and chased with H_2O_2 or left untreated.

(C) Time course measurement of MFI of macrophages when pulsed with MitoPY1 and an infected with MRSA and monitored over time via flow cytometry.

(D) Immunoblots of IRE1α and GAPHD from cell lysates from NT-control and IRE1α KO macrophages.

(E) MFI of IRE1α KO macrophages and NT-control after labeling with MitoPY1 followed by MRSA infection and analyzed after 4h.

(F) MFI of macrophages labeled with MitoPY1 for 1h and infected with MRSA for 4h in the presence and absence of mROS scavenger NecroX-5.

(G) Percent of intracellular MRSA killing by macrophages in the presence and absence of NecroX-5. Percent killing was calculated by the following formula [1 - ($CFU_{\text{indicated time points}}$ / $CFU_{1h\ pi}$)] X 100, which represents the percent difference in CFU at indicated time point relative to 1h pi.

MFI of MitoPY1 quantification was determined by using FlowJo software, representing a geometric mean. MFI of each condition was subtracted from MFI obtained from unstained cells. Graphs are presented as mean of n≥3 independent experiments +/- SD. pValue: * < 0.05, **< 0.01 and ***< 0.001.

Figure 2. TLR signaling controls mH_2O_2 accumulation in the bacterial phagosome

(A) Representative fluorescent microscopy images of macrophages when pulsed with MitoPY1 (green) for 1h and chased with beads (red) or live and dead MRSA-mcherry (red) infection for 4h. Images were acquired by an Olympus IX-70 inverted live-cell fluorescence microscope and analyzed by MetaMorph imaging software.

(B) Quantification of mean fluorescent intensity (MFI) of MitoPY1 associated with macrophage phagosomes using imageJ software. Phagosomes were defined by the area in the cell where red fluorescent beads or MRSA are localized.

(C) Time lapse imaging of macrophages pulsed with MitoPY1 for 1h (green) and then infected with MRSA-mCherry (red). Time clock Minutes : Seconds.

(D) Live microscopy images of wild type (WT) and TLR 2/4/9 deficient (TLR2/4/9 KO) macrophages pulsed with MitoPY1 (green) for 1h and then infected with MRSA-mCherry (red) for 4h.

(E) Quantification of MFI of MitoPY1 associated with phagosomes from WT and TLR2/4/9 deficient macrophages using similar criteria as in panel B.

Graphs represent averages of MitoPY1 MFI from at least 305 phagosomes pooled from at least three independent experiments. Error bars represent standard error of the mean (SEM). pValue: * < 0.05 and **** < 0.0001.

Figure 3. Infection induced Parkin-dependent MDVs contribute to MRSA killing

(A) Confocal microscopy representative images of macrophages stimulated with beads (red) or infected with MRSA (Red) for 4h and stained with Tom20 (green) antibody. Images were acquired by Leica TCS SP8 scanning confocal microscope and deconvoluted using Huygens essential software. Right panel are processed images after subtraction of Tom20 positive large objects (surface area > 0.4 μm^2).

(B) Quantification of Tom20 positive smaller objects (surface area < 0.4 μm^2) per macrophage when stimulated with beads or infected with MRSA. Decovoluted confocal images were processed by Huygens essential software using the following criteria; 10% threshold, 10% seed and garbage of 50. Tom20 positive objects with surface area larger than 0.4 μm^2 were filtered out and the remaining objects were enumerated per cell basis. Graphs represent means of at least 107 cells pooled from three independent experiments +/- SEM.

(C) Representative confocal microscopy images of WT and Parkin deficient (*Park2*$^{-/-}$) macrophages infected with MRSA for 4h. Images were processed using similar criteria as in panel A.

(D) Quantification of Tom20 positive smaller objects (surface area < 0.4 μm^2) from WT and Parkin deficient macrophages infected with MRSA. Confocal microscopy images processed as in panel B and presented as mean +/- SEM from at least 135 cells pooled from three independent experiments.

(E) Flow cytometry mean fluorescence intensity (MFI) of WT and Parkin deficient macrophages when pulsed with MitoPY1 for 1h and infected with MRSA for 4h. Data represented as geometric mean of n≥3 independent experiments +/- SD.

(F) Representative live fluorescence wide-filed microscopy images of WT and Parkin deficient macrophages pulsed with MitoPY1 (green) for 1h and infected with MRSA-mCherry (red). Images were acquired at 4h pi and processed by MetaMorph software.

(G) Ratiometric measurement of MitoPY1 fluorescent intensity of phagosome relative to total cellular fluorescent intensity (MFI-phagosome/MFI-cell). Phagosomes were defined by the area of the cell where the red fluorescent MRSA-mCherry are located.

(H) Percent of MRSA intracellular killing by WT and Parkin deficient macrophages was quantified by the following formula [1 - ($CFU_{\text{indicated time points}}$ / $CFU_{1h\ pi}$)] X 100, which represent the percent difference in CFU obtained at indicated time point relative to 1h pi. Data are presented as mean of n≥3 independent experiments +/- SD.

(I) Percent of MRSA intracellular killing by RAW264.7 macrophages when treated with control DMSO or Dynamin-related protein 1 (Drp1) selective inhibitor (Mdivi-1, 25 μM).

pValue: * < 0.05, **< 0.01, ***< 0.001 and ****< 0.0001.

Figure 4. Parkin is essential for immunity during MRSA subcutaneous infection

(A) Bacterial burden in skin abscesses from male and female wild-type (WT) and Parkin deficient C57BL/6 mice (*Park2*$^{-/-}$) infected subcutaneously with 10^7 CFU of MRSA for 3 days. Horizontal lines represent the mean. Data are pooled from two independent experiments.

(B-C) KC or IL1β cytokine levels in skin abscess homogenate of WT and *Park2*$^{-/-}$ mice. Cytokine levels were quantified by ELISA. Graph bars represent mean of n=13 WT and 12 *Park2*$^{-/-}$ mice pooled from 2 independent experiments. Error bars represent standard error of the mean (SEM).

pValue: * < 0.05, ** < 0.01 and **** < 0.0001.

Figure 5. Blocked of phagolysosome fusion enhances MDV mediated mH_2O_2 killing

(A) Processed confocal microscopy representative images of RAW264.7 macrophages infected with MRSA-mCherry (red) for 4h and stained for Tom20 (green). Images were acquired by Leica TCS SP8 confocal scanning microscope and deconvoluted using Huygens essential software. Images were processed using the following setting; Threshold: 10%, Seed: 10% and Garbage: 50. Tom20 positive large objects (surface area > 0.4 μm^2) were subtracted from the original images to define Tom20 positive small objects.

(B) Quantification of Tom20 positive small objects (MDVs) per macrophage during MRSA-mCherry infection in the presence and absence of Bafilomycin A1. See detail in figure 3A. Data are presented as mean of at least 110 phagosomes from each condition pooled form three independent experiments +/- SEM.

(C) Representative live wide-field microscopy images of RAW264.7 macrophages pulsed with MitoPY1 (green) and chased 4h post MRSA-mCherry (red) infection in the presence of Bafilomycin A1 (Baf A1, 100 nM) or controlled solvent (DMSO).

(D) Mean fluorescent intensity (MFI) of MRSA-containing phagosomes when macrophages were being pulsed with MitoPY1 (green) and chased 4h post MRSA-mCherry (red) in the presence and absence of Bafilomycin A1 (Baf A1, 100 nM). Data represent mean of at least 321 phagosomes of each condition pooled from three independent experiments +/- SEM.

(E) Percent of MRSA intracellular killing by RAW264.7 macrophages treated with Bafilomycin A1 (Baf A1, 100 nM) or control solvent (DMSO). Percent killing was quantified by the following formula $[1 - (CFU_{\text{indicated time points}} / CFU_{1h\ pi})] \times 100$, which represent the percent difference in CFU obtained at indicated time point relative to 1h pi. Graph bars represent mean of $n \geq 3$ independent experiments +/- SD.

(F) Transmission electron microscope images of macrophage MRSA-containing phagosomes. Macrophages are infected with MRSA for 4h in the presence and absence of Bafilomycin A1 (Baf A1, 100 nM). Images were acquired by using JEOL JEM-1400 Plus transmission electron microscope. Arrows indicate vesicles that are localized inside the phagosomes.

587 pValue: ** < 0.01 and **** < 0.0001.

588

589 **Figure 6. Sod2 is the mitochondria payload delivered to phagosome to promote**
590 **mH_2O_2 killing**

591 (A) Representative confocal microscopy images of RAW264.7 macrophages infected
592 with MRSA (blue) or stimulated with bead (blue) for 4h and stained for Lamp1 (red) and
593 Sod2 (green). Images were acquired using Leica TCS SP8 confocal scanning
594 microscope and deconvoluted using Huygens essential software.

595 (B) Confocal microscopy images of a magnified area of macrophages where bead or
596 MRSA-containing phagosome is localized. Images in the right panel are the resulted
597 images after subtracting large Sod2 positive objects (surface area > 0.4 μm^2) from left
598 panel images.

599 (C) Quantification of Sod2 positive small objects (surface area < 0.4 μm^2) of 2 μm^2 area
600 of the cell where beads or MRSA are localized. Large Sod2 positive objects were
601 filtered out of the deconvoluted images and the number of remained Sod2 positive
602 objects were enumerated per 2 μm^2 areas of the cell where phagsosomes were
603 localized. Data represent mean of at least 60 phagosomes from three independent
604 experiments.

605 (D) Immunoblots of cell lysate from RAW264.7 macrophages stably transduced with
606 lentivirus-encoded shRNA for non-target (NT-Control) or Sod2 (Sod2 KD), probed with
607 an anti-Sod2 antibody or anti-Actin antibody as a loading control.

608 (E) Mean fluorescent intensity (MFI) of shRNA stably knockdown Sod2 (Sod2 KD) or
609 non-target (NT-Control) macrophages after pulsed with MitoPY1 and chased 4h post
610 MRSA infection. Samples were acquired by flow cytometry and analyzed by FlowJo
611 software. MFI represent geometric mean of of n≥3 independent experiments +/- SD.

612 (F) Intracellular MRSA killing by Sod2 knockdown (Sod2 KD) and non-target (NT-
613 Control) macrophages was quantified by using the following formula [1 - (CFU $_{\text{indicated time}}$
614 $_{\text{points}}$ / $CFU_{1h\ pi}$)] and expressed as percentage. Graph bars represent mean of percent
615 killing of n≥3 independent experiments +/- SD.

616 pValue: * < 0.05, *** < 0.001 and **** < 0.0001.

617

618 **STAR Methods**

619

620 **CONTACT FOR REAGENT AND RESOURCE SHARING**

621 Reagents and resources can be obtained by directing requests to the Lead Contact,
622 Mary O'Riordan (oriordan@umich.edu)

623

624 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

625

626 **Mice**

627 Wild-type C57BL/6 and *Park2*$^{-/-}$ are purchased from the Jackson Laboratory. *Tlr2/4/9*$^{-/-}$
628 have been described previously (Abuaita et al. 2015). All mice were maintained
629 according to an approved protocol in the Unit for Laboratory Animal Medicine (ULAM)
630 facilities at the University of Michigan Medical School.

631

632 **Cells**

633 Primary bone marrow derived macrophages (BMDMs) were prepared by flushing
634 mouse femurs in DMEM supplemented with 100 units/ml of Pen/Strep. Cells were
635 differentiated by incubation in BMDM medium (50% DMEM, 2 mM L-glutamine, 1 mM
636 sodium pyruvate, 30% L929-conditioned medium, 20% heat-inactivated fetal bovine
637 serum (FBS), 55 μM 2-mercaptoethanol, and Pen/Strep). L-929 and HEK 293T cells
638 were cultured in MEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate,
639 1mM Non-essential amino acid (NEAA), 10 mM HEPES, and 10% heat-inactivated
640 FBS). RAW 264.7 cells were cultured in RPMI 1640 containing 2 mM L-glutamine and
641 10% heat-inactivated FBS. All cells were incubated at 37ºC in 5% CO_2.

642

643 **Bacterial infections**

644 USA300 LAC, a community associated methicillin-resistant *Staphylococcus aureus*
645 strain (MRSA) and its isogenic strain harboring p*SarA*-mCherry plasmid (MRSA-
646 mCherry) (Boles and Horswill, 2008), were maintained at −80°C in LB medium
647 containing 20% glycerol. All strains were cultured in tryptic soy agar (TSA, Becton
648 Dickinson), and selected colonies were grown overnight at 37ºC with shaking (240 rpm)
649 in liquid tryptic soy broth. Bacteria were pelleted, washed and re-suspended in PBS.
650 The bacterial inoculum was estimated based on OD_{600}, and verified by plating serial
651 dilutions on TSA plates to determine colony forming units (CFU). Macrophages were
652 infected at a multiplicity of infection (MOI) of 20 in culture medium without antibiotic for
653 45 minutes. Infected macrophages were washed three times with PBS and incubated in

654 medium containing 100 µg/ml of gentamicin to kill extracellular bacteria for 15 minutes.
655 Media was exchanged with media containing 50 µg/ml of gentamicin for the remaining
656 time of the experiments.
657
658 **METHOD DETAILS**
659
660 **Macrophage bactericidal activity**
661 Macrophages were seeded in a 24-well tissue culture treated plate at a density of 1.5 X
662 10^5 cell/well. The next day, macrophages were infected with MRSA (MOI of 20). The
663 number of intracellular bacteria was determined by washing infected macrophages with
664 PBS, lysing with 0.1% NP-40, and enumerating bacterial CFUs via serial dilution on
665 agar plates. The percentage of killed MRSA was calculated by the following formula [1 -
666 ($CFU_{\text{indicated time points}}$ / $CFU_{1h\ pi}$)] X 100, which represents the percent difference between
667 CFU at indicated time points relative to 1h pi. Where indicated, macrophages were pre-
668 incubated with NecroX-5 (10 µM), Bafilomycin A1 (100 nM) or Mdivi-1 (25 µM) for 30
669 minutes before infection, and all inhibitors were maintained throughout the experiment.
670
671 **Mouse infection**
672 Subcutaneous MRSA infection was performed as previously described (Tseng et al.,
673 2011). Male and Female C57BL/6 mice and *Park2*$^{-/-}$ were shaved on the right flank.
674 Mice were inoculated with 10^7 bacteria in 100 µl of PBS subcutaneously on the shaved
675 area of the skin using a 27 gauge needle. Mice were sacrificed on day 3 post-infection
676 and skin abscesses were excised, weighed and homogenized in PBS. Total CFU per

677 mouse abscess were enumerated by serial dilution and plating on TSA agar. Total CFU
678 was converted to CFU/mg of tissue weight. Cytokines were quantified by ELISA at the
679 University of Michigan ELISA core and converted to pg/mg of tissue weight.
680
681 **Cellular mROS measurement**
682 Macrophages were plated in 60 mm non-treated dishes and treated with 10 μM MitoPY1
683 (TOCRIS) or 10 μM MitoSOX (Life Technology) for 1 hour. Macrophages were washed
684 three times with media and only when indicated, macrophages were treated for 30
685 minutes with NecroX-5 (10 μM) or control solvent prior to infection with MRSA (MOI of
686 20). For positive controls, macrophages were treated with 100 μM hydrogen peroxide
687 or 10 μM Antimycin A for 1 hour to induce the oxidation of MitoPY1 or MitoSOX,
688 respectively. Macrophages were subjected to flow cytometry and data were analyzed
689 with FlowJo software. The mean fluorescence intensity for each condition was
690 determined as the geometric mean.
691
692 **Phagosomal mROS measurement**
693 Macrophages were plated in 35 mm glass bottom dishes (MatTek). The next day,
694 macrophages were treated with MitoPY1 (10 μM) for 1 hour, washed three times with
695 media and stimulated with inert red fluorescent beads or infected with live or dead
696 (inactivated by paraformaldehyde) red fluorescent MRSA harboring p*SarA*-mCherry
697 (MOI of 20). Macrophages were imaged in Ringer buffer (155 mM NaCl, 5 mM KCl, 1
698 mM MgCl2.6H2O, 2 mM NaH2PO4.H2O, 10 mM HEPES, and 10 mM Glucose) with an
699 Olympus IX70 inverted live-cell fluorescence microscope. Fluorescence images were

700 further processed by MetaMorph imaging software. For quantification of mROS
701 association with phagosomes, the mean fluorescence intensity of MitoPY1 at the
702 phagosome area was measured by ImageJ. Phagosomal regions in the cell images
703 were defined by the location of red fluorescent beads or bacteria. For BMDM
704 experiments, a ratiometric mean fluorescence intensity of the phagosomal area over the
705 mean fluorescence intensity of the cell was calculated. This was done because Park2
706 deficient macrophages have higher global MitoPY1 fluorescence intensity when
707 compared to WT macrophages prior to infection.
708
709 **Confocal microscopy**
710 Macrophages were seeded onto microscope cover glass and infected with MRSA (MOI
711 of 20) or stimulated with fluorescent beads. Cells were fixed at 4h pi with 3.7%
712 paraformaldehyde at room temperature for 20 minutes and permeabilized with PBS
713 contain 0.1% Triton X-100 for 15 minutes. MRSA were stained using chicken anti-
714 protein A antibody conjugated to biotin (Abcam ab18598) in staining buffer (PBS, 0.1%
715 Triton X-100, 5% BSA, and 10% normal goat serum). Host proteins were stained using
716 mouse anti-Sod2 (Abcam ab110300, clone 9E2BD2), Rat-anti-Lamp1 (DSHB, clone
717 1D4B), and anti-Tom20 (Santa cruz, FL145). Secondary antibodies (goat anti-mouse
718 (Alexa-488), goat anti-Rat (Alexa-594) and Streptavidin (Alexa-405) were used
719 according to manufacturer's procedure. Cover glasses were mounted on microscope
720 slides using Prolong Diamond (Life Technology). Cells were imaged using a Leica TCS
721 SP8 confocal microscope and deconvoluted using Huygens essential software by
722 scientific volume imaging using the following criteria; Threshold (10%), Seed (10%) and

723 Garbage Volume (50). To define MDVs, large objects (surface area larger than 0.4 μm^2)
724 were filtered out from the mitochondrial fluorescence labeled channel and the number of
725 remaining objects per cell were recorded.
726
727 **Transmission electron microscopy**
728 Macrophages were infected with MRSA (MOI of 20) in the presence of Bafilomycin A1
729 (100 nM) or control DMSO. Infected macrophages were fixed at 4h pi with 2.5%
730 glutaraldehyde for at least 1h at room temperature, then overnight at 4°C. For immuno-
731 gold staining, infected macrophages were stained for Tom20 prior to fixation with
732 glutaraldehyde according to manufacturer's procedure (AURION). Briefly, cells were
733 fixed with 3.7% paraformaldehyde at room temperature for 20 minutes and
734 permeabilized with Sorenson's buffer containing 0.1% Triton X-100 for 15 minutes. Cells
735 were blocked with the AURION blocking solution (AURION-BSA-c) and stained using
736 primary anti-Tom20 antibody (Santa Cruz, FL145) and secondary goat anti-rabbit ultra-
737 small gold antibody (AURION). Silver stain enhancement was carried out by using the
738 AURION R-GENT SE-EM according to reagent protocol (AURION). Glutaraldehyde
739 fixed samples were washed with Sorenson's buffer 3-times before post-fixing in 2%
740 osmium tetroxide in Sorenson's buffer for 1h at room temperature. Samples were
741 washed again 3-times with Sorenson's buffer, then dehydrated through ascending
742 concentrations of ethanol, treated with propylene oxide, and embedded in EMbed 812
743 epoxy resin. Semi-thin sections were stained with toluidine blue for tissue identification.
744 Selected regions of interest were ultra-thin sectioned to 70 nm and post stained with

745 uranyl acetate and Reynolds lead citrate. Sections were examined using a JEOL JEM-
746 1400 Plus transmission electron microscope (TEM) at 80 kV.
747
748 **Generation of RAW264.7*Δlre1-α* and RAW264.7 shRNA stable knockdown cells**
749 The generation of lentivirus for CRISPR-Cas9 knockout and shRNA knockdown was
750 done by using HEK293T packaging cells, which were grown in DMEM with 10% FBS.
751 The virus particles were produced by transfecting the cells with the TRC shRNA
752 encoded plasmid (pLKO.1) or guided RNA (gRNA) encoded plasmid (lentiCRISPRv2)
753 along with the packaging plasmids (pHCMV-G, and pHCMV-HIV-1) (Kulpa et al., 2013)
754 using FUGENE-HD transfection reagent (Promega). Media was changed after 24h and
755 virus particles were collected after 72h post-transfection. A total of 2 ml of medium
756 containing virus were concentrated ten-fold by ultracentrifugation at 24,000 rpm for 2h
757 at 4ºC and used to transduce RAW264.7 cells. Transduced cells were selected with
758 puromycin (3 µg/ml). The mouse Sod2 specific shRNA plasmid with the sense
759 sequence of (GCTTACTACCTTCAGTATAAA) and the non-target control shRNA
760 plasmid were purchased from Sigma-Aldrich. The efficiency of knockdown was
761 monitored by immunoblot analysis using anti-Sod2 antibody (Santa Cruz). Anti-Actin
762 antibody was used as a loading control (Fisher Scientific). The mouse IRE1-α specific
763 gRNA sequence of (CTTGTTGTTTGTCTCGACCC) and the non-target gRNA control
764 sequence of (TCCTGCGCGATGACCGTCGG) were cloned into lentiCRISPRv2
765 according to the Feng Zhang lab protocol (Sanjana et al., 2014). Single clones of
766 RAW264.7*Δlre1-α* were isolated and confirmed by the absence of IRE1-α protein by
767 immunoblot using anti-IRE1-α antibody (clone 14C10, Cell Signaling). Anti-GAPDH

antibody was used as a loading control (Santa Cruz). RAW264.7Δ*Ire1-*α clone was also confirmed by absence of its endonuclease activity when cells were treated with endoplasmic reticulum stress inducer Thapsigargin (5 μM) by *xbp1* splicing assay (Figure S1) as previously described (Abuaita et al., 2015).

Cell death assay

Cell death was measured by flow cytometry using SYTOX green dead cell stain according to manufacturer's protocol (Life Technology). Briefly, macrophages were incubated with SYTOX green dead cell stain (30 nM) in HBSS for 20 minutes at room temperature prior to flow cytometry analysis using 488 excitation and 530/30 emission. Digitonin (0.01%, Sigma Aldrich) was used as a positive control to permeabilize the plasma membrane. The percent of SYTOX positive cells was determined by gating against mock unstained cells.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed using Excel 2016 and Student's unpaired two-tailed t-test was applied. The mean of at least three independent experiments was presented with error bars showing standard deviation (SD) or standard error of the mean (SEM), which is indicated in figure legends. *P* values of less than 0.05 were considered significant and designated by: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001. All statistically significant comparisons within experimental groups are marked.

DATA AND SOFTWARE AVAILABILITY

RAW data are available upon request, which should be directed to the Lead Contact. There was no proprietary software used in this study.

ADDITIONAL RESOURCES

mCherry-Mito-7 plasmid was used under material transfer agreement (MTA).

Supplemental Information

Figure S1. IRE1α endonuclease activity is absent in RAW264.7ΔIRE1α macrophage cell line in response to ER Stressor, Thapsigargin

RT-PCR analysis of *Xbp1* mRNA splicing in single clone of RAW 264.7 macrophages stably transduced with IRE1α specific guided RNA (gRNA) or non-target (NT-control). Macrophages were treated with 5 μM thapsigargin (TG) to induce IRE1α endonuclease activity or control DMSO. RT-PCR products were digested with *PstI* endonuclease. Because unspliced *Xbp1* mRNA contains a *PstI* site within the 26 spliced region, the digested RT-PCR products yield two smaller fragments representing the unspliced (U) *Xbp1* and one larger fragment representing the spliced (S) *Xbp1*. RT-PCR image is representative of n≥3 independent experiments.

Figure S2. TLR2/4/9 signaling is dispensable for MRSA-induced mH_2O_2 but is essential for macrophage bactericidal activity

(A) Mean fluorescent intensity (MFI) of wild-type (WT) and *Tlr2/4/9* triple knockout (TLR2/4/9 KO) macrophages that were loaded with MitoPY1 and infected with MRSA. Macrophages were subjected to flow cytometry at 4h pi and collected data were analyzed by FlowJo software for geometric mean.

(B) Percent of MRSA intracellular killing by WT and TLR2/4/9 KO macrophages. Macrophage killing efficiency was calculated by using the following formula [1 - ($CFU_{\text{indicated time points}}$ / $CFU_{1h\ pi}$)] X 100, which represent the percent difference in CFU obtained at indicated time point relative to 1h pi. Data are expressed as percentage.

Graph bars represent mean of of n≥3 independent experiments +/- SD. pValue: ** < 0.01 and **** < 0.0001.

Figure S3. Tom20 positive MDVs are defined by small objects that are stained with Tom20

(A) Representative confocal images of MRSA infected and beads internalized macrophages that stained for Tom20. Images were acquired by Leica TCS SP8 confocal scanning microscope and deconvoluted using Huygens essential software. Images were processed using the following setting; threshold: 10%, Seed: 10% and Garbage: 50. Right panel, Tom20 positive large objects (surface area > 0.4 μm^2) were subtracted from left panel Images.

(B) Quantification the number of Tom20 positive objects per beads internalized or MRSA infected macrophage that fall into indicated range of surface size bins.

833 Discontinuous line was drawn to point out the surface area size bin smaller than 0.4
834 μm^2, which was chosen to quantify Tom20 small objects (MDVs).

835

836 **Figure S4. Tom20 positive vesicles are localized in the lumen of MRSA**
837 **phagosomes**

838 Transmission electron microscopy representative mages of MRSA infected
839 macrophages when treated with Bafilomycin A1 (Baf A1, 100 nM) or control DMSO.
840 Tom20 was stained with immune-gold particles followed by silver enhancement. Left
841 panel; images showing whole cell, middle panel; magnified images showing
842 mitochondria, right panel; images showing MRSA phagosome. Arrows were drawn to
843 indicate Tom20 positive gold particles present in MRSA phagosome lumen.

844

845 **Figure S5. Macrophage Sod2 is localized with Tom20**

846 Representative confocal microscopy images of RAW264.7 macrophages were stained
847 with Sod2 and Tom20. Images were acquired with Leica TCS SP8 confocal scanning
848 microscope and deconvoluted using Huygens essential software. Pearson Correlation
849 Coefficient (PCC) was determine on the deconvoluted images using Huygens essential
850 software and presented as mean of at least 230 cells +/- SD pooled from three different
851 experiments.

852

Figure S6. Knockdown macrophage Sod2 increases mitochondria superoxide production without interfering with host cell death during MRSA infection

(A) Representative histogram plots are shown when macrophages were pulsed with MitoSOX for 1h and chased 4h after stimulated with Antamycin A (10 μM), infected with MRSA at MOI of 20 or left untreated (Mock). Percent of MitoSOX positive cells was determined by gating against unstained cells. Percent of MitoSOX high cells was determined by gating against the MitoSOX first peak.

(B) Quantification of the percentage MitoSOX high from panal A. Data represent the percentage of cells with MitoSOX high relative to total MitoSOX positive cells.

(C) Percent of live macrophages was determined by Sytox green dead cell staining. NT-control and Sod2 KD macrophages were infected with MRSA for 4h (MOI of 20), treated with 0.01% Digitonin (Digitonin) to induce cell death or left untreated (Mock). Cells were stained with 30 nM of Sytox green in HBSS buffer and subjected to flow cytometry. Data are analyzed by FlowJo software. Percent of Sytox green positive cells were determined by gating against live cell peak.

Graph bars represent mean of n≥3 independent experiments +/- SD. pValue: * < 0.05 and ** < 0.01.

Movie S1. mH_2O_2 accumulates in MRSA-containing phagosome

Time-lapse movie of RAW264.7 macrophage after being pulsed with mitochondria-targeted H_2O_2 fluorescent sensor, MitoPY1 (green fluorescent) for 1h and infected with MRSA-mCherry (Red fluorescent). Images were acquired using inverted IX-70 Olympus

875 live fluorescent microscope and analyzed using MetaMorph imaging software. Time

876 clock Minutes : Seconds.