

1 Macrophage aggregates-like induced structures are flexible organizing platforms for immune  
2 signaling

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5 Marie-Eve Charbonneau, Vedhika Raghunathan and Mary X.D. O’Riordan\*

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7 Department of Microbiology and Immunology, University of Michigan Medical School, Ann  
8 Arbor, Michigan, United States of America.

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10 \*Corresponding author: [oriordan@umich.edu](mailto:oriordan@umich.edu)

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13 Short running title: ALIS as flexible immune signaling platforms

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17 SUMMARY

18 Charbonneau et al. demonstrate that ubiquitin- and p62-containing cytosolic ring-shaped structures  
19 induced by bacterial infections, microbial ligands and cytosolic double-stranded DNA contain  
20 context-dependent immune regulators, revealing an important insight on the cellular architecture  
21 required to coordinate signal transduction in macrophage.

22

23 ABSTRACT

24 Macrophages adopt a pro-inflammatory phenotype in response to environmental challenges in a  
25 process that often coincides with the formation of transient cytosolic p62/SQSTM1 inclusions  
26 containing ubiquitinated proteins in structures known as aggresome-like induced structures  
27 (ALIS). Although described as stress-induced inclusions that accumulate aggregated proteins, little  
28 direct evidence supports their hypothesized structural role in the context of immune stimulation.  
29 Here, we showed that these structures in primary macrophages are induced by multiple microbial-  
30 based ligands, including exposure to cytosolic double-stranded DNA. Rather than accumulating  
31 aggregated proteins, we observed that ubiquitinated proteins form a ring-shaped structure around  
32 the perimeter of these circular foci. We identified that different microbial stimuli induced the  
33 formation of ubiquitin-positive foci with distinct characteristics and we observed selective  
34 recruitment of context-dependent immune regulators. Our findings are consistent with a model  
35 where these ubiquitin-containing structures act as adaptable organizing centers for innate immune  
36 signaling.

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38 Macrophages are essential for control of homeostasis, tissue repair, and inflammation and are a  
39 part of the front-line defense against invading pathogens (Wynn et al., 2013, Murray and Wynn,  
40 2011). Macrophages recognize microbial challenge through a variety of pattern-recognition  
41 receptors (PPRs), including Toll-like receptors (TLRs), nucleotide-binding domain, leucine-rich  
42 repeat-containing proteins (NLRs), the AIM2-like receptors (ALRs), and several DNA sensors  
43 such as cGAS, the DNA-sensing enzyme cyclic guanosine monophosphate-adenosine  
44 monophosphate synthase (Fitzgerald and Kagan, 2020, Kieser and Kagan, 2017). Although these  
45 immune receptors share no structural similarities, a unifying principle governs downstream  
46 signaling events. Indeed, activation of these receptors leads to the formation of large oligomeric  
47 complexes that allow signal transduction and generation of inflammatory mediators such as  
48 cytokines and cell-intrinsic defense mechanisms, including the expression of interferon (INF)-  
49 stimulated genes (Kagan et al., 2014, Fitzgerald and Kagan, 2020). The formation of these  
50 supramolecular organizing centers (SMOCs) involves the oligomerization of adaptor proteins and  
51 the recruitment of specific effector proteins, which leads to the formation of localized punctate  
52 structures in the cells. Examples of SMOCs include the Myddosome, which consist of a TLR  
53 sensor, the downstream adaptors MyD88 and TIRAP as well as protein kinases of the IRAK  
54 family, and the inflammasome, which includes an NLR protein, the ASC adaptor, and a member  
55 of the caspase protein family (Kagan et al., 2014). The concentration of signaling components into  
56 specific subcellular structures is thought to enable an effective immune response by increasing the  
57 threshold for effector protein activation and signal amplification (Fitzgerald and Kagan, 2020).  
58 Despite these recent advances in our knowledge of the cell response to microbial sensing, more  
59 work is needed to better understand the spatiotemporal organization of signaling molecules that  
60 link the initial SMOCs formation and the downstream long-lasting cellular responses, including  
61 changes in transcription, translation, and metabolism.

62

63 A type of punctate cytosolic structure that was described in immune cells upon stimulation with  
64 TLR ligands or during infection with bacterial pathogens such as *Listeria monocytogenes* and  
65 *Mycobacterium tuberculosis* is the aggresome-like induced structure (ALIS) (Szeto et al., 2006,  
66 Liu et al., 2012, Fujita et al., 2011, Canadien et al., 2005). These structures are solely characterized  
67 by the presence of ubiquitinated proteins and the multi-functional adaptor protein p62/SQSTM1.  
68 Similar structures were observed in many cell types in response to proteotoxic stresses including

69 treatment with an endoplasmic reticulum (ER) stress inducer, a protein translation inhibitor, and  
70 exposure to reactive oxygen species (ROS) (Vasconcellos et al., 2016, Liu et al., 2012, Jena et al.,  
71 2018). Accordingly, ALIS are described as structures that accumulate misfolded ubiquitinated  
72 proteins upon cellular stress, when the degradative pathways such as the proteasome and the  
73 autophagy machinery are overwhelmed. In this model, p62 serves as a scaffolding component  
74 required to package these aggregated ubiquitinated proteins. Of note, ALIS puncta are distinct  
75 from aggresomes as their formation is independent of the microtubule network and they are not  
76 surrounded by a vimentin cage (Szeto et al., 2006). Although first described in dendritic cells upon  
77 inflammatory stimulation (Lelouard et al., 2002), the information available regarding these  
78 structures was mostly acquired by studying diverse cell types exposed to a variety of cell stressors  
79 that are not related to immune responses. Therefore, a detailed analysis of the structure and  
80 components present in these cytosolic puncta, specifically in the context of innate immune  
81 signaling, remains to be determined.

82

83 Multiple lines of evidence suggest that ALIS might be more than depots of aggregated proteins in  
84 immune cells. First, these foci form extensively after TLR stimulation, which would suggest that  
85 triggering an immune response in macrophages is concomitant with the accumulation of a large  
86 number of misfolded proteins that need to be contained into specific depots for future degradation.  
87 However, little evidence supports that TLR signaling induces accumulation of misfolded proteins  
88 or that prevention of ALIS formation following immune stimulation correlates with cellular  
89 toxicity in macrophages. Second, p62 is an important protein for homeostatic cell functions besides  
90 its role in packaging aggregated proteins (Moscat et al., 2016, Sanchez-Martin et al., 2019). This  
91 multivalent adaptor protein can self-assemble and form liquid-like bodies or droplets, a process  
92 enhanced by binding to ubiquitin chains, which is consistent with the co-occurrence of these two  
93 proteins in cytosolic condensates (Sun et al., 2018). Multiple reports have shown that p62 droplets  
94 can act as signaling hubs for protein activation, including major components of signaling pathways  
95 like the nuclear factor erythroid 2-related factor 2 (Nrf2), the mechanistic target of rapamycin  
96 complex 1 (mTORC1), the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B)  
97 and to promote caspase-8 activation (Jin et al., 2009, Park et al., 2013, Sanchez-Martin et al., 2020,  
98 Duran et al., 2011, Kehl et al., 2019). Together, these studies highlight the potential for p62  
99 containing structures to organize signaling pathways instead of promoting protein degradation.

100 Lastly, multiple bacterial pathogens prevent the formation of punctate structures containing  
101 ubiquitinated proteins during infection. Indeed, the *Salmonella enterica* serovar Typhimurium type  
102 III secretion system effector SseL, a bacterial deubiquitinase, prevents the formation of ubiquitin-  
103 positive cytosolic puncta during infection. (Mesquita et al., 2012). Similarly, in a type IV secretion  
104 system-dependent manner, *Legionella pneumophila* and *Brucella abortus* actively prevent the  
105 formation of ALIS, although the specific effector proteins involved in this process remain to be  
106 identified (Ivanov and Roy, 2009, Salcedo et al., 2008). Taken together, these observations open  
107 up new possibilities regarding the nature of ALIS foci and their potential implication in  
108 macrophage immune defenses.

109

110 Here, we show that the formation of ubiquitin-containing cytosolic structures occurs in  
111 macrophage in response to various immune stimuli, which include TLR stimulation, bacterial  
112 infection, and exposure to cytosolic double-stranded DNA (dsDNA). Furthermore, we show that  
113 while ubiquitinated proteins and ubiquitin-binding proteins like p62 are shared elements of these  
114 structures, other components are context-specific and depend on the signal triggering the immune  
115 response. We also provide evidence that these structures selectively recruit key components of  
116 signaling pathways, including proteins involved in the NF- $\kappa$ B pathway or the type I interferon  
117 response. Therefore, our findings are consistent with the idea that these cytosolic ubiquitin-  
118 containing foci might be similar to the SMOCs and act as subcellular sites in macrophages where  
119 innate immune signaling occurs. A better understanding of these ubiquitin-containing structures  
120 from a cell biology angle provides an important insight into how macrophages perform signal  
121 transduction in a regulated manner to promote productive immune responses.

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123

## 124 RESULTS

125

126 **LPS stimulation induces the formation of ring-shaped cytosolic ubiquitin- and p62-positive**  
127 **structures in primary macrophages.** Formation of ubiquitin (Ub)-enriched cytosolic structures  
128 occurs in response to multiple cellular stresses, including oxidative and proteotoxic stresses,  
129 leading to the suggestion that these foci are misfolded proteins waiting for degradation (Liu et al.,  
130 2012, Vasconcellos et al., 2016, Szeto et al., 2006, Jena et al., 2018). Similar structures are also

131 observed following immune stimulation of dendritic cells and macrophages, but the nature of these  
132 Ub-containing foci in the context of immune stimulation remains elusive. To directly address this  
133 question, we treated primary bone marrow-derived macrophages (pBMDM) with 10 ng/ml of  
134 lipopolysaccharides (LPS) for 6h and stained them for p62 and polyubiquitinated proteins.  
135 Approximately 86% of cells stimulated with LPS displayed 2 or more foci, while the number of  
136 Ub-positive foci in these cells ranged from 2 to 20 with an approximate average of 6 foci per cell  
137 (Fig. 1A). These LPS-induced Ub-positive foci have an average size of around  $0.94 \mu\text{m}^2$  (Fig. 1B).  
138 We observed similar results using concentrations of LPS ranging from 0.1 ng/ml up to 10 ng/ml  
139 (Fig. S1A), suggesting that a more physiological concentration of 0.1 ng/ml of LPS is enough to  
140 trigger Ub-containing foci formation in macrophage. The ubiquitin protein has seven lysine  
141 residues that can be linked to other ubiquitin molecules. Globally, K48-linked ubiquitin chains are  
142 associated with proteasomal degradation, whereas K63-linked chains are important for protein  
143 trafficking, stability, and many signaling processes, including innate immune signaling (Yau and  
144 Rape, 2016). By staining macrophages with antibodies specific for K48- and K63-polyubiquitin  
145 chains, we observed that these cytosolic puncta are enriched in both polyubiquitin linkages. These  
146 observations suggest that ubiquitinated proteins contained in these structures might not all be  
147 targets for degradation but might rather play a functional or structural role (Fig. S1B). LC3, a  
148 marker of the autophagosome that binds directly to p62, is recruited to the Ub- and p62-containing  
149 foci (Levine et al., 2011, Fujita et al., 2011). We confirmed the recruitment of LC3 to the Ub-  
150 containing foci in pBMDM (Fig. S1C); however, the formation of these structures was independent  
151 of the classic autophagy machinery. Indeed, at 6h post LPS stimulation, there was no significant  
152 difference in the number of foci per cell when *Atg7<sup>fl/fl</sup>* LysM-Cre pBMDM were compared to  
153 *Atg7<sup>fl/fl</sup>* (WT) cells (Fig. S1D). We could, however, observe more Ub-positive foci in the cytosol  
154 of *Atg7<sup>fl/fl</sup>* LysM-Cre pBMDM compared to WT pBMDM at 24h post-stimulation with LPS,  
155 suggesting a possible role for the autophagy process in clearing these structures (Fig. S1E).

156  
157 ALIS were described as sites for accumulation of misfolded and aggregated proteins during stress,  
158 a role similar to that associated with aggresome formation (Szeto et al., 2006). In agreement, Ub-  
159 enriched foci observed during oxidative stress contain aggregated proteins (Jena et al., 2018). To  
160 directly test the assumption that ALIS induced by LPS treatment are depots of aggregated proteins,  
161 we used the reagent ProteoStat™, a widely used protein aggregate-specific molecular rotor dye

162 that intercalates into quaternary structures usually found in misfolded and aggregated proteins and  
163 emits fluorescence (Bershtein et al., 2013, Seo et al., 2016, Shen et al., 2011). Primary BMDM  
164 treated with MG132, a proteasome inhibitor (Lee and Goldberg, 1998), for 6h or 18h clearly  
165 showed strong fluorescence that co-localized with ubiquitinated proteins (Fig. 1C). The mean  
166 fluorescence intensity of ProteoStat™ was significantly higher after proteasome inhibition when  
167 compared to untreated cells, confirming the utility of this reagent in assessing protein aggregates.  
168 Macrophages treated with LPS showed accumulation of Ub-positive puncta, but these structures  
169 were not labeled by ProteoStat™, suggesting that LPS stimulation is not associated with the  
170 accumulation of misfolded and aggregated proteins (Fig. 1C). Other types of cytosolic foci are  
171 observed in cells after proteotoxic stress, including stress granules, which are membrane-less  
172 compartments containing mainly untranslated mRNA and RNA-binding proteins (Protter and  
173 Parker, 2016). As stress granules shared similar properties compared to the Ub-containing  
174 structures observed in LPS-stimulated macrophages, we tested for the presence of a stress granule  
175 component, G3BP, in foci formed following immune stimulation. Co-staining of pBMDM for  
176 ubiquitinated proteins and G3BP showed that Ub-containing foci formed in response to LPS were  
177 distinct from the stress granules induced by heat-shock (Fig. 1D).

178  
179 To better define the nature of the Ub-containing foci observed in response to LPS stimulation, we  
180 examined their structure by high-resolution confocal imaging. We observed that the Ub- and p62-  
181 containing foci commonly formed a ring-shaped structure (Fig. 1E-F). By analyzing the staining  
182 pattern of an individual puncta, we found that ubiquitinated proteins localized mainly on the edge  
183 of the spherical focus whereas p62, although enriched on the edge, also stained the region toward  
184 the center of the spherical structure (Fig. 1E). Detailed analysis of these LPS-induced Ub-positive  
185 foci showed a mean circularity (sphericity) of 0.94 (Fig. 1G). The consistent high circularity score  
186 of these foci might suggest that they behave similarly to membrane-less biomolecular condensates  
187 with liquid-liquid phase separation properties (Snead and Gladfelter, 2019, Wang and Zhang,  
188 2019, Alberti et al., 2019). Moreover, the absence of staining for ubiquitinated proteins inside these  
189 foci is in good agreement with the idea that these structures are not just depots of aggregated  
190 proteins, in which case we would expect the entire foci to be labeled for proteins tagged with  
191 ubiquitin molecules. Taken together, our results unveil that these Ub-containing puncta observed

192 in LPS-stimulated macrophages are organized structures, a characteristic reminiscent of other  
193 higher-order signaling complexes, like SMOCs, involved in immune signaling.

194

195 **LPS induces ALIS formation through a transcriptional program, with a minor contribution**  
196 **from the Nrf2 pathway.** SMOC assembly occurs rapidly after recognition of a ligand, as  
197 exemplified by the formation of the myddosome within minutes after LPS stimulation (Fitzgerald  
198 and Kagan, 2020, Bonham et al., 2014). TLR dimerization allows the recruitment of the  
199 components already available and needed for formation of the signaling platform. ALIS assembly  
200 may occur through a distinct mechanism, as the earliest time we could observe Ub-positive  
201 structures in pBMDM was at 4h post-stimulation with LPS, suggesting a possible requirement for  
202 transcriptional events (Fig. S2A). In agreement with this hypothesis, the formation of Ub-  
203 containing foci in stimulated macrophages was blocked by treatment with actinomycin D, an  
204 inhibitor of mRNA synthesis, or with cycloheximide, an inhibitor of protein synthesis (Fig. S2B).  
205 Likewise, ALIS formation in response to LPS was abolished in TLR2/4/9<sup>-/-</sup> immortalized bone  
206 marrow-derived macrophages (iBMDM) (Fig. S2C). Formation of Ub-containing foci in the  
207 context of oxidative stress, induced either by treatment with hydrogen peroxide or exposure to  
208 heme, is driven by the transcription factor NF-E2-related factor (Nrf2), a master regulator of the  
209 cellular response against oxidative stress (Szeto et al., 2006, Vasconcellos et al., 2016, Jena et al.,  
210 2018). However, ALIS formation induced by the autophagy inhibitor 3-MA is independent of the  
211 Nrf2 transcriptional response (Wenger et al., 2012), suggesting a disparity in the dependency for  
212 Nrf2 activation depending on the signal initiating the formation of these Ub-containing cytosolic  
213 structures. To further investigate the role of Nrf2 signaling for the formation of Ub-containing  
214 structures in our experimental model, we treated WT and Nrf2<sup>-/-</sup> pBMDM with LPS for 6h and  
215 analyzed foci formation. Although the number of Ub-containing foci per cell was significantly  
216 lower, these structures could still be observed in approximately 74% of Nrf2<sup>-/-</sup> macrophages (Fig.  
217 S2D). In agreement, 73% of cells treated with the reactive oxygen species scavenger *N*-  
218 acetylcysteine (NAC) had Ub-positive foci after LPS stimulation, although a lower number of foci  
219 per cell was detectable (Fig. S2E). Thus, our results suggest that activation of macrophages using  
220 LPS triggers the formation of Ub-containing cytosolic structures in a TLR- and transcription-  
221 dependent manner, and but does not require activation of the major cellular oxidative pathway.

222



223 **Distinct microbial ligands induce the formation of ubiquitin and p62-positive foci.** We used  
224 LPS as an archetypal microbial TLR ligand to understand the detailed structure of the Ub-  
225 containing foci. Macrophages, however, recognize microbial challenge through a variety of  
226 pattern-recognition receptors beyond TLR4. To determine the relevance of ALIS formation after  
227 immune stimulation, we first treated cells with Pam3CSK4, a synthetic triacetylated lipopeptide,  
228 which is a potent TLR1/TLR2 heterodimer agonist that mimics bacterial lipoproteins found in both  
229 Gram-positive and Gram-negative bacteria. As with LPS, Pam3CSK4 treatment induced the  
230 formation of Ub-positive structures in a TLR2/4/9-dependent manner (Fig. 2A). We also observed  
231 that infection by two Gram-positive human pathogens, methicillin-resistant *Staphylococcus aureus*  
232 (MRSA) and *L. monocytogenes*, induced the formation of cytosolic structures containing  
233 ubiquitinated proteins (Fig. 2B-C). MRSA induction of ALIS was dependent on TLR signaling as  
234 foci formation was abolished in TLR2/4/9<sup>-/-</sup> iBMDM (Fig. 2B). However, foci were observed in  
235 TLR2/4/9<sup>-/-</sup> iBMDM infected with *L. monocytogenes*, although at a significantly lower number  
236 compared to WT cells (Fig. 2C). *L. monocytogenes* is an intracellular pathogen that replicates in  
237 the cytosol of infected cells and therefore can trigger the activation of multiple cytosolic innate  
238 immune pathways including the Type I interferon response (Woodward et al., 2010). DNA release  
239 from *L. monocytogenes* induces IFN- $\beta$  secretion in a cGAS-, IFI16-, and STING-dependent  
240 manner (Hansen et al., 2014). Similarly, detection of the bacterial second messenger c-di-AMP  
241 secreted by *L. monocytogenes* promotes STING activation and the Type I Interferon response  
242 (Parvatiyar et al., 2012). As ALIS formation was observed independently of TLR2/4/9 in response  
243 to *L. monocytogenes* infection, we investigated the possibility that DNA sensing might be a trigger  
244 for the formation of Ub-positive cytosolic foci in macrophages. Indeed, transfection of G3-YSD,  
245 a palindromic DNA sequence that self-hybridized to form a Y-form short dsDNA and acts as a  
246 cGAS agonist (Herzner et al., 2015), as well as transfection of an immune-stimulatory DNA (ISD),  
247 resulted in the formation of Ub-positive foci in macrophages (Fig. 2D). A detailed analysis of the  
248 foci formed after infection with *L. monocytogenes* or transfection of dsDNA showed ring-shaped  
249 structures comparable to the foci observed after LPS stimulation (Fig. S3A-B). These Ub-positive  
250 foci could also be detected using a p62 antibody, highlighting again the similarity with the LPS-  
251 induced foci (Fig. S3C). Notably, we observed that Ub-positive structures often formed around the  
252 transfected DNA, as highlighted by DAPI staining (Fig. S3C). Although G3-YSD is a cGAS  
253 agonist, cGAS was not required for the formation of the Ub-containing foci. Indeed, cGAS<sup>-/-</sup>

254 pBMDM transfected with G3-YSD displayed a similar number of foci compared to WT pBMDM  
255 (Fig. S4A-B). Similarly, the downstream signaling adaptor molecule STING was not required for  
256 foci formation in response to dsDNA (Fig. S4C-D), suggesting that recognition of cytosolic  
257 dsDNA is a signal that triggers the formation of ubiquitinated protein-containing foci  
258 independently of the cGAS/STING signaling pathway. Taken together, our results are consistent  
259 with the idea that the formation of cytosolic ubiquitin and p62-positive structures is a common  
260 response to immune stimulation in macrophages.

261

262 **Different microbial stimuli induce the formation of ubiquitin structures with different**  
263 **properties.** We showed that multiple immune stimuli induced the formation of Ub- and p62-  
264 containing structures similar to those observed in LPS-treated cells. Treatment of cells with the  
265 TLR1/2 agonist Pam3CSK4 induced the formation of Ub-positive foci with a similar size range  
266 and abundance when compared to LPS stimulation (Fig. 2A and Fig. 3A), suggesting a common  
267 response downstream of TLRs engagement that triggers the assembly of foci with similar structural  
268 features. However, some differences in the Ub-positive foci structures were easily noticeable when  
269 other microbial ligands were used for stimulation. Indeed, macrophages transfected with dsDNA  
270 had a lower number of Ub-containing foci per cell compared to LPS stimulation (Fig. 2D).  
271 Moreover, the average sizes of the foci induced by either infection with *L. monocytogenes* (average  
272 size of 1.5  $\mu\text{m}^2$ ) or dsDNA transfection (average size of 2.1  $\mu\text{m}^2$ ) were significantly larger  
273 compared to LPS-induced puncta (average size of 0.94  $\mu\text{m}^2$ ) (Fig. 3B-C). The circularity of the  
274 foci was also variable depending on the immune triggers, from a circularity value of 0.94 for LPS  
275 stimulation to 0.80 for *L. monocytogenes* infection and 0.82 for dsDNA transfection (Fig. 3D). Of  
276 note, the Ub-containing foci observed after *L. monocytogenes* infection or transfection with  
277 dsDNA were not labeled using the Proteostat™ dye, which is consistent with the idea that these  
278 foci are not representing an accumulation of aggregated proteins (Fig. 3E).

279

280 As the structural features of these Ub-containing foci seem to be shaped by the stimuli used to  
281 induce their formation, we decided to look at the requirement for the major known component of  
282 ALIS, the adaptor protein p62. Using p62<sup>-/-</sup> pBMDM, we found that this protein is required for the  
283 formation of Ub-containing foci in response to LPS stimulation (Fig. 3F), in agreement with  
284 previous reports (Fujita et al., 2011, Liu et al., 2012). However, p62<sup>-/-</sup> pBMDM infected with *L.*

285 *monocytogenes* displayed a significant number of Ub-containing foci per cell, although these  
286 structures assembled less efficiently when compared to WT pBMDM. Although p62 is observed  
287 in the Ub-containing foci formed following dsDNA transfection (Fig. S3C), their formation can  
288 occur independently of p62. Indeed, the formation of Ub-containing structures was similar in WT  
289 and p62<sup>-/-</sup> pBMDM transfected with either G3-YSD or ISD (Fig. 3F). Another characteristic of the  
290 LPS-induced Ub-containing foci is the presence of the autophagy-associated protein LC3. To  
291 determine if this association is a unifying principle, we stained pBMDM infected with *L.*  
292 *monocytogenes* or transfected with dsDNA with an LC3 specific antibody. We observed that *L.*  
293 *monocytogenes* infection triggered the association of LC3 to the Ub-containing foci, whereas LC3  
294 was absent from the structures generated after dsDNA exposure (Fig. 3G). Altogether, we showed  
295 here that although these Ub-containing foci that are induced by immune stimulation share some  
296 common features, the structural requirement and the composition of these condensates might be  
297 dependent on the input signal.

298

299 **Selective recruitment of innate immune regulators in ubiquitin-positive structures.** Besides  
300 the presence of ubiquitinated proteins, p62, and in some contexts, LC3, nothing is known about  
301 the contents of these Ub-containing cytosolic structures. We have shown that various immune  
302 triggers can lead to the formation of structures containing ubiquitinated proteins with different  
303 characteristics and morphology. In this context, it is tempting to propose the idea that these  
304 structures might play a role in immune signal transduction that will differ depending on the stimuli.  
305 To address this possibility, we directly assessed the spatial distribution of some major immune  
306 regulators downstream of TLR4 and cGAS. TLR4 engagement by LPS triggers the assembly of  
307 the Myd88-containing complex the myddosome, which in turn promotes the activation of  
308 downstream protein kinases including the IKK complex and the MAP kinase (MAPK) p38 and  
309 their respective transcription factors NF- $\kappa$ B and AP1 (Fitzgerald and Kagan, 2020). A major  
310 modulator of the canonical NF- $\kappa$ B pathway is NEMO (IKK $\gamma$ ), the regulatory subunit of the IKK  
311 activating complex (Maubach et al., 2017). pBMDM treated for 6h with LPS or transfected with  
312 the cGAS ligand G3-YSD were stained for ubiquitinated proteins and NEMO. We observed that  
313 approximately 31% of the Ub-containing foci were enriched for NEMO in response to LPS  
314 stimulation (Fig. 4A). Conversely, dsDNA transfection did not induce the relocation of NEMO to  
315 the Ub-positive structures, suggesting specificity in the components recruited to the Ub-containing

316 foci depending on the immune trigger. Similarly, we investigated the localization of the MAPK  
317 p38 and found that the activated phosphorylated form of p38 could be detected in nearly 60% of  
318 Ub-positive structures after LPS stimulation (Fig. 4B). In contrast to what we observed for NEMO  
319 localization, exposure to dsDNA seemed to also be a signal for recruitment of phosphorylated p38,  
320 as approximately 44% of the Ub-positive foci induced by G3-YSD transfection were also enriched  
321 for this protein. Engagement of TLR4 can also induce signaling through the Toll/IL-1 receptor  
322 (TIR) domain-containing adaptor protein, TRIF (Yamamoto et al., 2003, Fitzgerald et al., 2003).  
323 TRIF signaling occurs through the TANK binding-kinase 1 enzyme (TBK1) and the transcription  
324 factor IRF3 to trigger Type I interferon expression. Besides its role in TLR signaling, TBK1 is an  
325 essential component of the cGAS/STING signaling pathway. Indeed, downstream of dsDNA  
326 sensing by cGAS, STING interacts directly with TBK1 to promote IRF3 activation and the Type  
327 I interferon response (Motwani et al., 2019). In resting macrophages, we observed that the  
328 activated and phosphorylated form of TBK1 is present at a low level (Fig. 4C). However, upon  
329 stimulation of pBMDM with either LPS or dsDNA, staining for p-TBK1 showed high prevalence  
330 for this protein in cytosolic puncta that also contained ubiquitinated proteins (Fig. 4C). Besides  
331 TBK1, cGAS is a major contributor to the cellular response to cytosolic dsDNA. As we observed  
332 the presence of DAPI-positive molecules inside the Ub-containing structures upon dsDNA  
333 transfection (Fig. S2C), we decided to look directly at cGAS spatial localization following  
334 exposure. The localization of cGAS in resting macrophages was mainly nuclear (Fig. 4D), in  
335 agreement with a previous report (Volkman et al., 2019). However, upon dsDNA transfection, a  
336 shift in the spatial distribution for the cGAS signal was visible, with increased detection in the  
337 cytosol, especially in proximity to the Ub-containing structures. Indeed, nearly 50% of the  
338 ubiquitin foci were enriched for cGAS following dsDNA transfection. In LPS-stimulated cells,  
339 cGAS was observed at a low frequency in the Ub-containing foci, which is quite reminiscent of  
340 the selective presence of NEMO in the foci induced by LPS treatment. Our results, therefore,  
341 suggest that multiple immune stimuli triggered the formation of Ub-containing cytosolic foci, but  
342 the components observed in these structures are dependent on the nature of the stimuli. These  
343 observations raise the interesting possibility that these Ub- and p62-containing foci might be  
344 structural platforms for innate immune signaling and the signal inducing their formation dictates  
345 the recruitment of specific components.

346

347 **Ligand recognition shapes the structural organization of the ubiquitin-containing foci.** We  
348 have reported that cytosolic dsDNA induces the formation of Ub-containing foci that seems to  
349 encircle the cytosolic transfected dsDNA (Fig. S3C). This observation might suggest a correlation  
350 between the recognition of cytosolic dsDNA molecules and the formation of selective circular-  
351 shaped structures. The unpaired guanosine trimers at the ends of each strand of the dsDNA  
352 molecule G3-YSD are required for the activation of cGAS (Herzner et al., 2015). A derivative of  
353 this dsDNA molecule was previously described where the guanosine trimers were replaced by  
354 cytidine trimers to create the Y-shaped dsDNA C3-YSD. Although cGAS can bind to C3-YSD,  
355 this interaction results in a lower *in vitro* production of cGAMP and a level of INF- $\alpha$  secretion  
356 similar to untreated cells (Herzner et al., 2015). We confirmed that the transfection of primary  
357 macrophages with G3-YSD, but not with C3-YSD, induced the secretion of INF- $\beta$  (Fig. 5A).  
358 Although the transfection of C3-YSD activated only weakly cGAS, this dsDNA molecule induced  
359 the formation of Ub-positive structures in the cytosol of macrophages (Fig. 5B). However, these  
360 structures are quite different from the ones observed following G3-YSD transfection (Fig. 5B).  
361 Indeed, C3-YSD transfection induced the formation of larger and irregularly shaped Ub-enriched  
362 structures, as shown by the area and circularity measurements (Fig. 5C-D). A closer analysis of  
363 the Ub-containing foci showed that while G3-YSD induced ring-shape structures, exposure to C3-  
364 YSD generated irregular-shaped foci that are filled with ubiquitinated proteins (Fig. 5E). These  
365 observations could suggest that DNA recognition is a first step in the formation of condensates  
366 containing ubiquitinated proteins, however, activation of the sensor is required for the assembly  
367 of higher-ordered structures. Taken together, our results are consistent with the idea that the ring-  
368 shaped structural organization is required for efficient downstream immune signaling.

369

370

## 371 DISCUSSION

372

373 Multiple conditions or stresses have been linked to the presence of cytosolic structures that contain  
374 ubiquitinated proteins. The best-studied example is the aggresome, which is a large assembly of  
375 aggregated and ubiquitinated proteins that are localized at the perinuclear region near the MTOC  
376 and occurs primarily when the function of the ubiquitin-proteasome system is altered (Kopito,  
377 2000). Another example is the ALIS foci, which contain ubiquitinated proteins but possess

378 different properties compared to the aggresome, like their size, their cellular localization, and the  
379 absence of a vimentin cage surrounding them, among others (Szeto et al., 2006). These  
380 condensates were seen in response to multiple stresses, including blocks in protein translation  
381 generating truncated proteins unable to fold properly, treatment with heavy metals, exposure to an  
382 oxidative environment, and immune stimulation (Jena et al., 2018, Liu et al., 2012, Szeto et al.,  
383 2006, Vasconcellos et al., 2016). The current model explaining the formation of these structures,  
384 which relies mainly on observations obtained using cellular stressors that have in common to  
385 induce protein misfolding, suggests that p62 will recruit free cytosolic polyubiquitinated and  
386 unfolded proteins and assemble them into packages. Although the idea of generalizing these  
387 findings to all structures containing ubiquitinated proteins independent of the signal triggering  
388 their formation is highly attractive, a better understanding of the relationship between the element  
389 triggering the formation and the components recruited to these foci is required to fully understand  
390 the nature of these structures.

391  
392 In this study, we used a low concentration of LPS as a physiologic immune trigger to directly study  
393 the structural features of these Ub-containing inclusions in the context of immune stimulation. We  
394 could observe that foci generated in response to immune stimulation shared structural similarities  
395 with the foci generated following proteotoxic stress (Fujita et al., 2011, Jena et al., 2018, Liu et  
396 al., 2012, Szeto et al., 2006, Vasconcellos et al., 2016), including the presence of ubiquitinated  
397 proteins and Ub-binding proteins like the adaptor protein p62. However, our study provides  
398 multiple lines of evidence suggesting that these Ub-containing foci cannot be considered as one-  
399 size-fits-all and multiple variations of these structures can be observed depending on the signal  
400 triggering their formation. Indeed, although the current model suggests that these foci are an  
401 assembly of misfolded proteins, we observed here that immune stimulation of macrophages is not  
402 associated with an increase in cytosolic protein aggregation. The presence of aggregated proteins  
403 in Ub-containing puncta was described in cells exposed to oxidative stress, which is a condition  
404 known to damage proteins and induced aggregation (Jena et al., 2018, Weids et al., 2016).  
405 However, macrophages are highly plastic cells that can rapidly adapt their function in response to  
406 an environmental challenge (Murray and Wynn, 2011, Wynn et al., 2013). As these cells are a part  
407 of the front-line defense against invading microbes, it is not surprising that sensing microbial  
408 presence is not associated with the overwhelming of the cellular degradation capacity and

409 formation of micron-size protein aggregates. We also found that immune stimulation resulted in  
410 the formation of ring-shaped structures with the ubiquitinated proteins localized on the edges of a  
411 structure, an observation at odds with the current model of protein aggregates. Indeed, amorphous  
412 protein aggregation described the abnormal and unordered association of misfolded proteins  
413 leading to larger aggregates (Hartl et al., 2011). In this scenario, we should observe ubiquitinated  
414 proteins all over the inclusions, not only on the edges. The third line of evidence comes from the  
415 result that the Nrf2 transcription factor is not essential for the formation of Ub-positive puncta in  
416 response to LPS stimulation, in good agreement with the assembly of ALIS foci independently of  
417 Nrf2 in response to the autophagy inhibitor 3-MA (Wenger et al., 2012). However, this observation  
418 differs from previous studies showing that the Nrf2 signaling pathway was required in response to  
419 oxidative stress as well as overnight treatment with a high and non-physiological concentration of  
420 LPS (Jena et al., 2018, Vasconcellos et al., 2016, Fujita et al., 2011), suggesting that the  
421 requirement for Nrf2 activity is context-dependent. Taken together, these observations are  
422 consistent with the idea that distinct signaling pathways can lead to the generation of comparable  
423 Ub-containing cytosolic puncta, but possibly serving context-dependent functions.

424

425 Although it was previously shown that LPS stimulation and intracellular bacterial infections were  
426 sufficient to trigger the formation of Ub-containing foci in macrophages (Liu et al., 2012, Canadien  
427 et al., 2005), the extent of signal that can instigate this response in macrophages was still ill-  
428 defined. Here, we showed that ligands from both Gram-positive and Gram-negative bacteria,  
429 infection with the bacterial pathogens *L. monocytogenes* and MRSA, as well as exposure to  
430 cytosolic dsDNA, a hallmark of multiple viral and bacterial infections, are signals for the formation  
431 of Ub-containing structures in macrophages. Even though these signals induced the formation of  
432 ring-shaped structures containing ubiquitinated proteins surrounding the edges of the foci, major  
433 differences could be observed regarding the number of foci per cell, their size, and their circularity.  
434 Moreover, the basic core components required for the formation of these structures is dependent  
435 on the signal triggering their formation. Indeed, LPS-induced foci are completely dependent on  
436 the presence of the adaptor protein p62 whereas Ub-positive foci can be observed in response to  
437 cytosolic dsDNA in cells deficient for p62. This observation is somehow reminiscent of the  
438 differential role for Nrf2 signaling depending on the trigger and reinforces the idea that the  
439 formation of these Ub-containing structures is context-dependent. More importantly, we also

440 observed that a strong agonist is required for the formation of the typical ring-shaped foci  
441 following dsDNA sensing, suggesting a direct link between the recognition of the immune stimuli,  
442 the formation of the Ub-containing structures, and the resulting immune response. Accordingly,  
443 we found a significant enrichment of multiple regulators of major innate immune signaling  
444 pathways downstream of either LPS stimulation or cytosolic dsDNA sensing in these Ub-  
445 containing structures. This recruitment occurs with a certain degree of specificity as NEMO, a  
446 major modulator of the canonical NF- $\kappa$ B pathway, was present only in foci formed downstream  
447 of LPS stimulation whereas cGAS was mainly observed in those triggered by dsDNA sensing.  
448 Taken together, our results are consistent with the idea that the cytosolic puncta containing  
449 ubiquitinated proteins generated after immune stimulation of macrophages are structural platforms  
450 containing context-dependent components of major immune signaling pathways.

451

452 An emerging concept in innate immunity is the assembly of large oligomeric and modular  
453 platforms downstream of microbial detection to regulate host defenses. The SMOCs, which  
454 included the myddosome, the triffosome, and the inflammasomes among others, are well-  
455 described examples of such complexes. These SMOCs shared the properties of assembling on  
456 membranous organelles and often required proteins containing death effector domains (DEDs),  
457 pyrin domains (PYRs), or caspase activation and recruitment domains (CARDs) that nucleates  
458 helical filament formation by homotypic protein-protein interactions (Kagan et al., 2014, Ha et al.,  
459 2020). Although this model for immune signaling is attractive, the SMOCs are not unique in their  
460 function of organizing centers for immune responses. Indeed, it was recently shown that  
461 multivalent interaction between cGAS and dsDNA induced the formation through phase separation  
462 of liquid-like cytosolic foci in which cGAS is concentrated to enhance the production of the second  
463 messenger cGAMP required for STING activation and the Type I interferon response (Du and  
464 Chen, 2018). Stress granules are another type of membrane-less organelle generated by liquid-  
465 liquid phase separation that plays the role of organizing platforms for innate immune signaling.  
466 Stress granules are dynamic biomolecular condensates that assemble in a context-dependent  
467 manner in response to multiple cellular conditions, leading to a variety of functions, from the  
468 alteration of mRNA translation and degradation to modulation of signaling pathways and antiviral  
469 responses (Alberti et al., 2019, Protter and Parker, 2016). Besides the long-known antiviral role of  
470 stress granules in blocking protein translation, multiple antiviral regulatory proteins, including



471 RIG-I, PKR, and RNase L, are recruited to these granules to promote their activation and efficient  
472 immune response (Manivannan et al., 2020, Onomoto et al., 2012, Reineke and Lloyd, 2015).

473

474 The Ub-containing structures observed here in macrophages might serve a function similar to the  
475 ones described for SMOCs or the antiviral stress granules. Ubiquitinated proteins and ubiquitin-  
476 binding proteins like p62 might act as a scaffold for the formation of these structures, either by  
477 forming oligomeric structures similar to what was observed for the assembly of SMOC complexes  
478 or by promoting a liquid-liquid phase separation. As only a few components of these Ub-  
479 containing structures are known, it is possible that a major protein, possibly modified through  
480 ubiquitination, can assemble into large helical structures required for the formation of micron-size  
481 foci in cells. Alternatively, these structures might assemble through phase separation into  
482 molecular condensates, where proteins can diffuse freely and retain their native conformation and  
483 activities. In agreement with this model, p62 was shown to induce liquid-liquid phase separation  
484 in a process that was dependent on the presence of ubiquitin chains (Sun et al., 2018). Undoubtedly,  
485 distinguishing between these two models will require further studies and might help shape our  
486 understanding of the mechanism behind the assembly of immune signaling complexes in  
487 macrophages. An interesting feature of these Ub-positive structures is related to their formation  
488 timeline. For comparison, the myddosome assembles minutes after engagement of TLR4 by LPS  
489 while these Ub-containing structures started being visible around 4h post-stimulation, suggesting  
490 that these two complexes might regulate different stages of the macrophage response to sustain  
491 LPS exposure. An alternative model that we cannot exclude to explain our observations is a  
492 function for these Ub-containing structures in the modulation of signaling pathway through the  
493 sequestration instead of activation of critical components, a suggested function for stress granules  
494 in the regulation of the RACK1, TORC1, and TRAF2-dependent signaling pathways (Arimoto et  
495 al., 2008, Kim et al., 2005, Takahara and Maeda, 2012). Future work aimed at identifying the  
496 components of the Ub-containing structures observed under immune stimulation will be required  
497 to better understand their function in the regulation of macrophage immune responses. In  
498 summary, our results are consistent with the idea that the cytosolic Ub-containing foci might be  
499 subcellular sites for regulation of innate immune signaling in macrophages.

500

501

502 MATERIALS AND METHODS

503

504 **Mice.** Mice were housed in specific pathogen-free facilities, maintained by the Unit for Lab  
505 Animal Medicine of the University of Michigan. Wild-type C57BL/6 mice (stock No: 000664),  
506 Nrf2<sup>-/-</sup> (stock No:017009), cGAS<sup>-/-</sup> (stock No: 026554), and Sting<sup>-/-</sup> (stock No: 025805) were  
507 purchased from Jackson Laboratories. The TLR2/4/9<sup>-/-</sup> femurs were a gift from T. Merkel (FDA)  
508 (Hassan et al., 2012). Femurs from male and female WT and p62<sup>-/-</sup> littermate mice were a gift from  
509 J. Moscat (Sanford Burnham Prebys Medical Discovery Institute) (Duran et al., 2004). The Atg7<sup>fl/fl</sup>  
510 and the Atg7<sup>fl/fl</sup> LysM-Cre femurs were a gift from Dr. J. A. Swanson (University of Michigan  
511 Medical School) (Komatsu et al., 2005, Hwang et al., 2012). Whenever possible, independent  
512 replicate experiments were done using cells isolated from different mice, including from male and  
513 female animals. This study was carried out following the recommendations in the guide for the  
514 care and use of laboratory animals of the National Institutes of Health and the protocol was  
515 approved by the committee on the care and use of animals of the University of Michigan.

516

517 **Primary cell culture and cell lines.** Bone-marrow derived macrophages (BMDM) were prepared  
518 by flushing mouse femurs in Dulbecco's modified Eagle's medium (DMEM) medium. Specific  
519 BMDM media containing 50% DMEM, 30% L929-conditioned medium, 20% heat-inactivated  
520 fetal bovine serum (FBS), 5% L-Glutamine, 1% sodium pyruvate, and 0.05% β-mercaptoethanol  
521 and 100 units/ml of penicillin-streptomycin (Pen/strep) was used for differentiation. Cells were  
522 fed fresh media after three days and incubated for an additional three days to complete the  
523 differentiation process. To generate immortalized BMDM (iBMDM), bone-marrow cells were  
524 transduced with the J2 retrovirus immediately after isolation and differentiated in macrophages as  
525 above (Gandino and Varesio, 1990). iBMDM were cultured in DMEM medium supplemented with  
526 10% FBS, 10% L929-conditioned medium, 5% L-Glutamine, 1% sodium pyruvate, 0.05% β-  
527 mercaptoethanol and Pen/Strep. Cells were incubated at 37°C with 5% CO<sub>2</sub>.

528

529 **Bacterial strains and culture condition.** *L. monocytogenes* 10403S was grown in brain heart  
530 infusion (BHI) broth statically at 30°C overnight. The community-acquired methicillin-resistant  
531 *Staphylococcus aureus* strain USA300 LAC harboring pSarA-GFP (MRSA-GFP) was cultured in  
532 tryptic soy medium (TSA, Becton Dickinson) overnight at 37°C with shaking (Boles et al., 2010).

533 Before each experiment, the bacteria were centrifuged, washed once, and diluted in PBS. The  
534 OD<sub>600</sub> was used to determine the inoculum.

535

536 **Macrophage infections and treatment.** pBMDM were seeded on 6-well plates onto microscope  
537 coverslips at a density of  $0.5 \times 10^6$  cells per well and incubated overnight. The next day, cells were  
538 treated in a final volume of 1 ml per well for 6h, unless specified. For immune stimulation,  
539 macrophages were treated with 10 ng/ml LPS (InvivoGen, tlr1-smlps), 100 ng/ml Pam3SCK4  
540 (InvivoGen, tlr1-pms) or transfected with 2 µg/ml of G3-YSD (InvivoGen, tlr1-ydna), 2 µg/ml of  
541 C3-YSD (InvivoGen, tlr1-ydnac), or 25 pmol of ISD (IDT, sequence:  
542 TACAGATCTACTAGTGATCTATGACTGATCTGTACATGATCTACA) using the  
543 transfection reagent Turbofect (ThermoFisher, R0531). Alternatively, cells were infected with *L.*  
544 *monocytogenes* at an MOI of 5 for 0.5h. After 3 PBS washes, cells were incubated in fresh media  
545 containing 10 µg/ml of gentamycin to kill extracellular bacteria. Similarly, cells were infected with  
546 MRSA USA300 at an MOI of 20 for 1h, followed by 3 PBS washes. Fresh media containing 100  
547 µg/ml of gentamycin was added for 20 minutes and replaced by media containing 10 µg/ml  
548 gentamycin for the remaining of the infection. For proteasome inhibition, pBMDM were treated  
549 for either 6h or 18h with 5 µM MG132 (Cayman Chemicals, S2619). Transcription and translation  
550 were blocked by treating the cells for 0.5h with either 1 µg/ml actinomycin D (Sigma-Aldrich,  
551 A9415) or 5 µg/ml cycloheximide (Sigma-Aldrich, C7698) before adding LPS at a final  
552 concentration of 10 ng/ml. The evaluation of the effect of reactive oxygen species was done by  
553 treating the cells for 6h with 10 mM of *N*-Acetyl-L-Cysteine (Millipore, 1009005).

554

555 **Immunofluorescence staining and confocal microscopy.** After treatment, cells were washed  
556 three times with PBS, fixed at room temperature for 20 minutes with 4% paraformaldehyde, and  
557 permeabilized with Tris-buffered saline (TBS) with 0.1% Triton X-100 for 10 minutes. All the  
558 staining was performed sequentially as strong cross-reactivity was observed, especially when  
559 using the guinea pig anti-p62 antibody (ARP, #03-GP62-C). Blocking for 45 minutes was  
560 performed at room temperature between each set of primary/secondary antibodies in TBS buffer  
561 containing 0.1% Triton X100, 3% bovine serum albumin (BSA), and 10% normal goat serum  
562 (ThermoFisher, #100000C). Incubations were done for 1h at room temperature in the staining  
563 buffer (TBS with 0.1% Triton X-100 and 3% BSA) for the primary antibodies and 0.5h for the

564 secondary antibodies. Coverslips were extensively washed between each step. The primary  
565 antibodies used are the mouse anti-polyubiquitinated conjugates (Enzo Life Sciences, BML-  
566 PW8805-0500), the guinea pig anti-p62 (ARP, 03-GP62-C), the rabbit anti-G3BP (Abcam,  
567 ab181150), the mouse anti-ubiquitin, Lys48-specific (Millipore, 05-1307), the mouse-anti-  
568 ubiquitin, Lys63-specific (Millipore, 05-1308), the rabbit anti-LC3 (MLB International, PM036),  
569 the rabbit anti-cGAS (Cell Signaling, 31659), the rabbit anti-IKK $\gamma$ /NEMO (Abcam, ab178872),  
570 the rabbit anti-phospho p38 (Thr180/Tyr182) (Cell Signaling, 4511), the rabbit anti-phospho  
571 TBK1 (Ser172) (Cell Signaling, 5483) and the rabbit *Listeria O* antisera (ThermoFisher, DF2300).  
572 Secondary antibodies used (all from ThermoFisher) are the Alexa Fluor-488 goat anti-mouse IgM  
573 (A21042), the Alexa Fluor-594 goat anti-mouse IgM (A21044), the DyLight-650 goat anti-mouse  
574 IgM (SA5-101053), the Alexa Fluor-488 goat anti-guinea pig IgG (A11073), the Alexa Fluor-488  
575 goat anti-rabbit IgG (A11034), and the Alexa Fluor-594 goat anti-rabbit IgG (A11037). DAPI was  
576 used to stained nucleic acids (ThermoFisher, D1306). Coverslips were mounted on microscope  
577 slides using the Prolong Gold mounting reagent (ThermoFisher, P36930). Images were taken on a  
578 Nikon A1 confocal microscope using a 60X objective and connected to the Nikon Elements  
579 software. The FIJI software was used for image processing (Schindelin et al., 2012). For  
580 quantification of the number and the size of the ubiquitin-containing foci, projections of stack  
581 confocal images representing 5  $\mu$ M thick sections of the cells were used. To label the foci, the  
582 analyze particle plugin was used with the size of particles set between 0.35 and 30  $\mu$ m<sup>2</sup> and the  
583 circularity factor above 0.3. Alternatively, the particle analysis was done on all particles with a  
584 circularity between 0 and 1 and the shape descriptor plugin was used to evaluate the degree of  
585 circularity. For quantification of the structures positive for ubiquitinated proteins and another  
586 component (cGAS, phospho-p38, phospho-TBK1, NEMO/IKK $\gamma$ , and LC3), single confocal  
587 sections were acquired. The mean fluorescence intensity (MFI) values inside the foci were  
588 calculated and compared to the MFI values of the entire cell cytosolic region. Each structure with  
589 an MFI 2-fold higher compared to the cytosolic background was considered positive.

590

591 **Protein extraction and immunoblotting.** pBMDM were seeded on a 6-well plate at a density of  
592  $1 \times 10^6$  cells per well and incubated overnight. After treatment of pBMDM as described above,  
593 whole-cell lysates were prepared using the following lysis buffer: 10 mM Tris-HCl pH8, 150 mM  
594 NaCl, 1 % NP-40, 10 mM EDTA pH8, 1 mM DTT, and 1X Roche protease inhibitors. Lysates

595 were incubated on ice for 15 minutes, diluted in 4X sample buffer (Biorad), quickly sonicated, and  
596 incubated at 95°C for 10 minutes. Samples were separated by SDS-PAGE and transferred to  
597 polyvinylidene fluoride membrane (PVDF, Millipore). Immunoblotting was performed according  
598 to the antibody manufacturers' instructions (cGAS: Cell Signaling, 31659; STING: Proteintech,  
599 19851-1-AP; Actin: Fisher, MS1295P1).

600

601 **Statistical analysis.** As indicated in figure legends, results represent the mean and the  
602 corresponding standard deviation of the mean for at least three independent experiments, unless  
603 specified. Statistical analysis was performed using GraphPad Prism 8 software and one-way  
604 analysis of variance (ANOVA) with Dunnett or Tukey's post-tests or unpaired two-tailed student's  
605 t-test were used, as indicated in each legend.

606

607

#### 608 SUPPLEMENTAL MATERIALS

609

610 **Fig. S1** investigates the type of ubiquitin-linkage observed in ALIS and the role of autophagy for  
611 ALIS formation. **Fig. S2** describes the role of transcriptional events for ALIS formation. **Fig. S3**  
612 examines the structural properties of Ub- and p62-positive foci induced by dsDNA transfection  
613 and infection with *L. monocytogenes*. **Fig S4** demonstrates that ALIS formation in response to  
614 cytosolic dsDNA is independent of cGAS and STING function.

615

616

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618

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630

631

## 632 AUTHOR CONTRIBUTIONS

633

634 M-E.C and V.R. performed experiments. M-E.C and M.X.O designed the experiments, analyzed  
635 the results, and wrote the manuscript.

636

637

## 638 DECLARATION OF INTEREST

639

640 The authors declare no competing interests.

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642

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### 833 ABBREVIATIONS

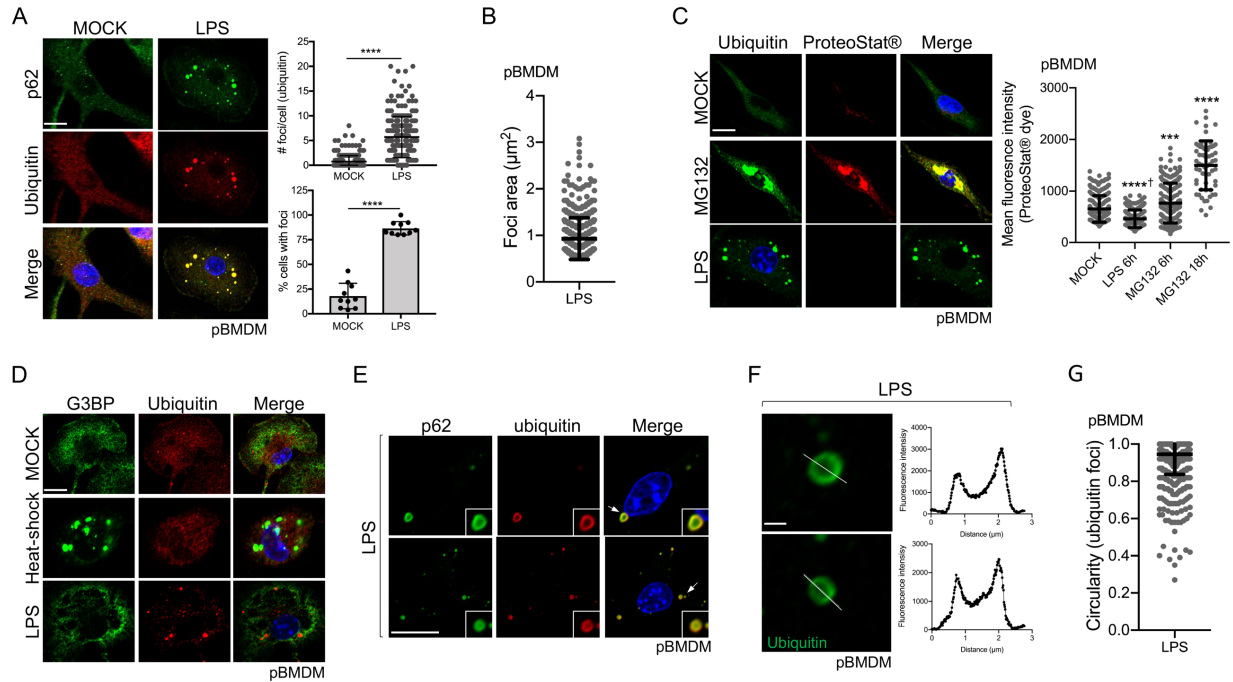
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835 ALIS, aggresome-like induced structures; dsDNA, double-stranded DNA; iBMDM, immortalized  
836 bone-marrow-derived macrophages; LPS, lipopolysaccharides; pBMDM, primary bone-marrow-  
837 derived macrophages; ROS, reactive oxygen species; SMOC, supramolecular organizing centers;  
838 TLR, Toll-like receptors; Ub, ubiquitin.

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### 841 FIGURES AND FIGURE LEGENDS



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843 **Figure 1: LPS stimulation induces the formation of cytosolic ring-shaped Ub- and p62-**

844 **positive structures.** *A.* Representative Confocal microscopy images of pBMDM left untreated or

845 stimulated for 6h with 10 ng/ml of LPS. Macrophages were stained using antibodies against p62

846 and polyubiquitinated proteins. Quantification was done using the particle analysis function of FIJI

847 to identify particles with a size between 0.5 and 15  $\mu\text{m}^2$  and with a circularity higher than 0.3. At

848 least 200 cells pooled from three independent experiments were used. *B.* Quantification of the foci

849 area ( $\mu\text{m}^2$ ) from pBMDM treated with 10 ng/ml of LPS for 6h. *C.* Representative images and

850 quantification of pBMDM left untreated, stimulated for 6h with 10 ng/ml of LPS, or treated with

851 the proteasome inhibitor MG132 for 6h or 18h and stained using an antibody against

852 polyubiquitinated proteins and the aggresome/aggresome-like specific dye ProteoStat<sup>TM</sup>. *D.*

853 Representative images of pBMDM left untreated, stimulated for 6h with 10 ng/ml of LPS or

854 incubated at 42°C for 1h (heat-shock), and stained with antibodies against polyubiquitinated

855 proteins and G3BP. *E.* Images representing a magnified area of pBMDM treated and stained as

856 described in *A.* The arrows indicate the region that is displayed in higher magnification. *F.*

857 Representative enlarged single confocal images showing the structures of a focus stained for

858 ubiquitinated proteins after 6h treatment with LPS 10 ng/ml. The fluorescence intensity was

859 calculated across the white line in the neighboring panel. *G.* Quantification of the foci circularity

860 (0 to 1) from pBMDM treated with 10 ng/ml of LPS for 6h. Graphs represent the mean and the

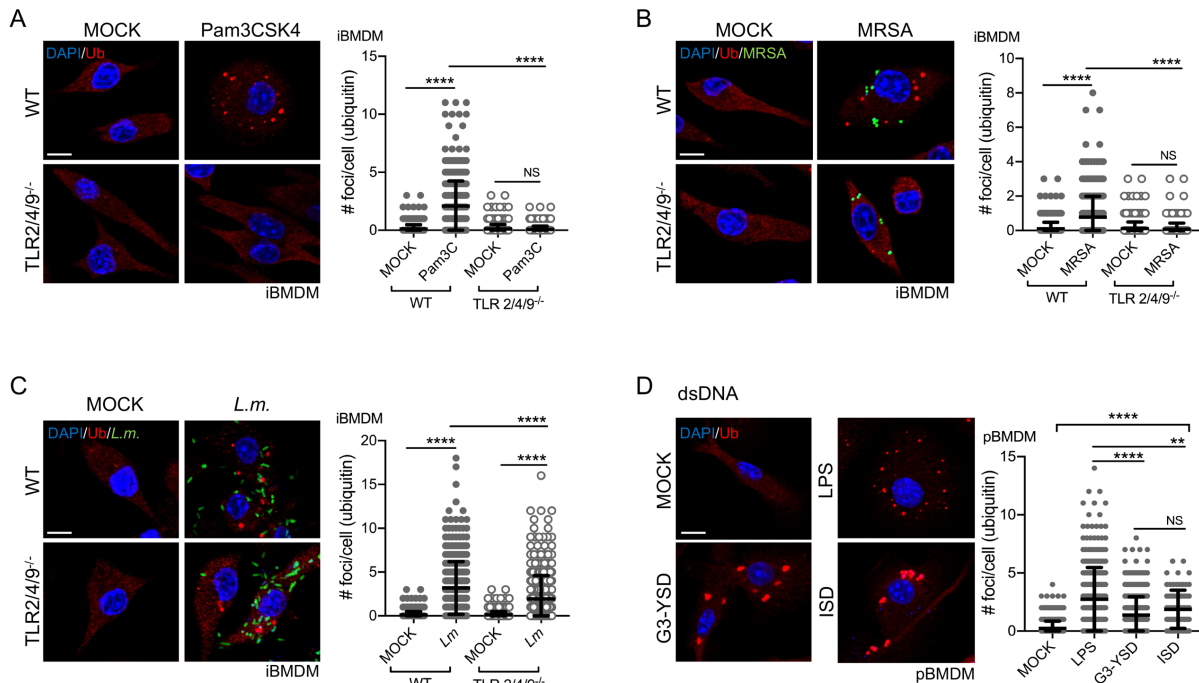
861 corresponding standard deviation of the mean and significant differences were calculated using

862 two-tailed Student's t-test (*A*) or one-way ANOVA with Tukey's post-test (*C*) (NS, not significant,

863 \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , † significantly lower). Scale bar of 10  $\mu\text{m}$  (*A, C, D, E*) or 1  $\mu\text{m}$  (*F*).

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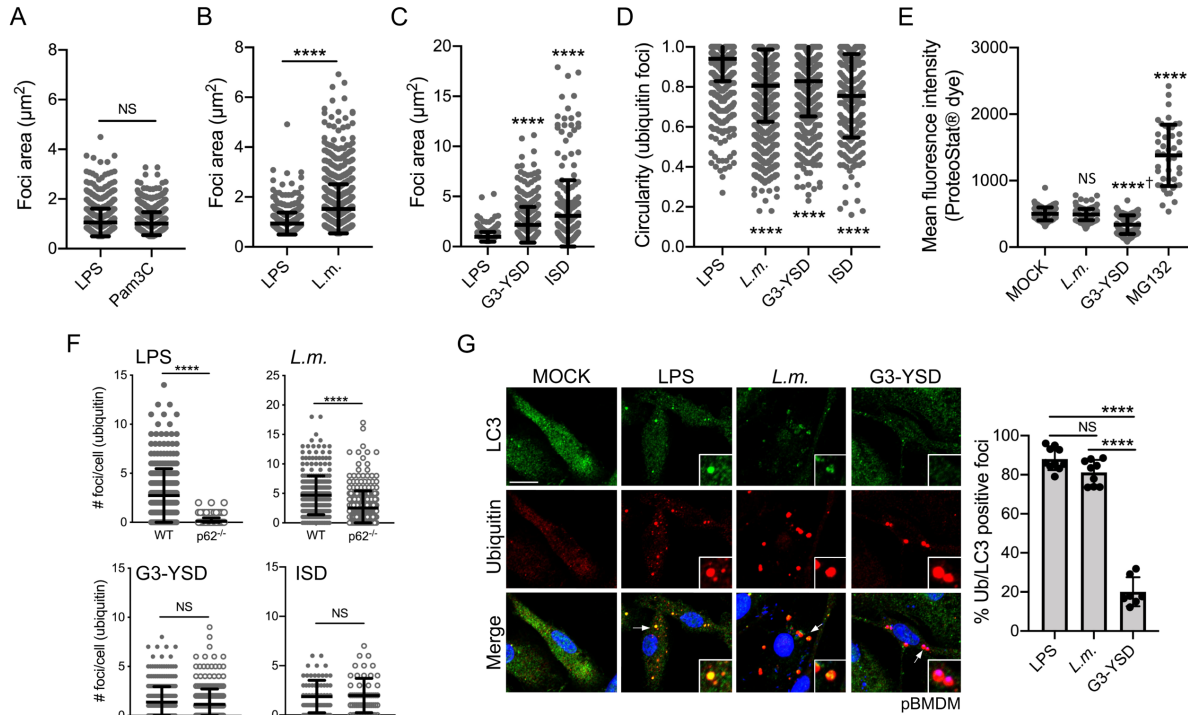
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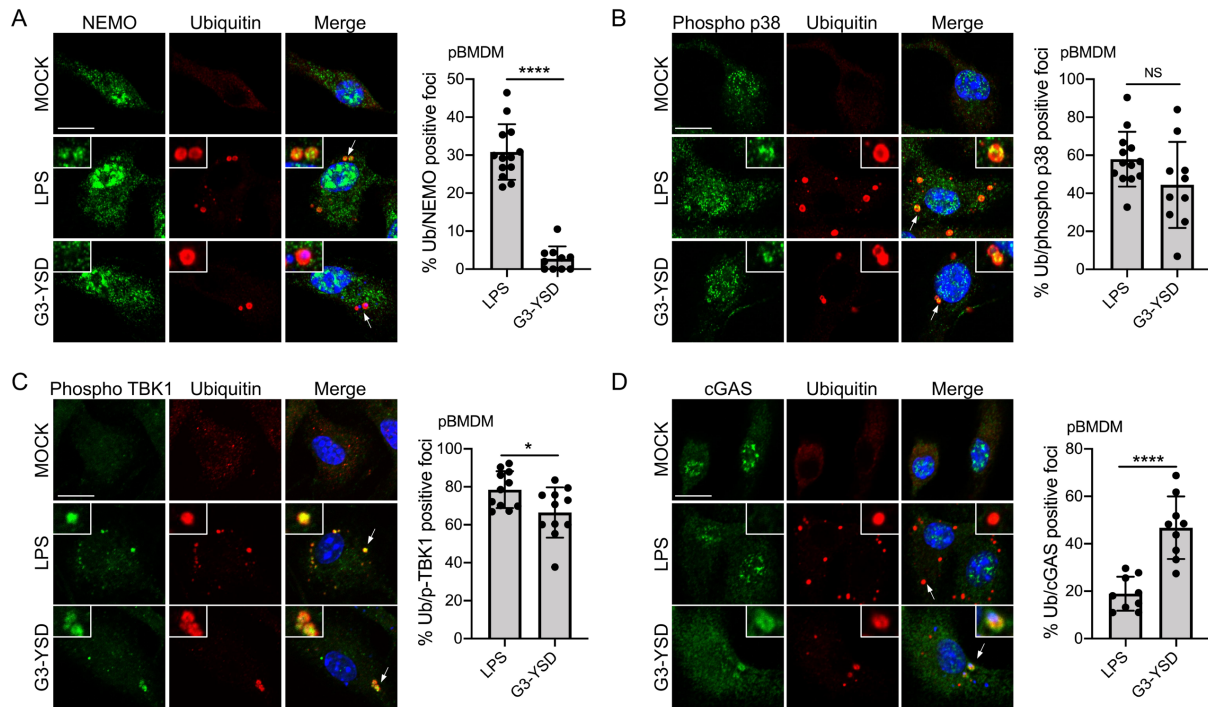
**Figure 2: Distinct microbial-based immune stimuli induce the formation of Ub- and p62-positive foci in macrophages.** *A.* WT and TLR2/4/9<sup>-/-</sup> iBMDM were left untreated or stimulated for 6h with 100 ng/ml of Pam3CSK4. *B.* WT and TLR2/4/9<sup>-/-</sup> iBMDM infected with MRSA-GFP at an MOI of 20 for 1h, washed and incubated for 6h in a medium containing gentamycin to kill extracellular bacteria. *C.* WT and TLR2/4/9<sup>-/-</sup> iBMDM were infected for 0.5h with *L. monocytogenes* at an MOI of 5, washed, and incubated for 6h in a medium containing gentamycin. *L. monocytogenes* was stained using specific *Listeria* O antisera. *D.* pBMDM were stimulated with 10 ng/ml of LPS, transfected with 2 μg/ml of G3-YSD, or transfected with 25 pmol of ISD for 6h. Staining (blue: DAPI; red: polyubiquitinated proteins; green: bacteria) and image processing was done as described in Figure 1. Graphs represent the mean and the corresponding standard deviation of the mean from three independent experiments using at least 200 cells per condition. Significant differences were calculated using one-way ANOVA with Tukey's post-test (NS, not significant, \*\* p < 0.01, \*\*\*\* p < 0.0001). Scale bar: 10 μm.



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 882 **Figure 3: Different microbial stimuli trigger the formation of Ub-containing structures with**  
 883 **different properties.** Quantification of the foci area (μm<sup>2</sup>) using the staining for polyubiquitinated  
 884 proteins from iBMDM treated with 10 ng/ml of LPS or 100 ng/ml of Pam3CSK4 for 6h (A),  
 885 infected for 0.5h with *L. monocytogenes* at an MOI of 5, washed and incubated for 6h in medium  
 886 containing gentamycin (B) or transfected with either 2 μg/ml of G3-YSD or 25 pmol of ISD for  
 887 6h (C). D. Quantification of the foci circularity (from 0 to 1) using the staining for  
 888 polyubiquitinated proteins from pBMDM treated with 10 ng/ml of LPS, transfected with 2 μg/ml  
 889 of G3-YSD or infected with *L. monocytogenes* as described above. E. Quantification of the mean  
 890 fluorescence intensity for the aggresome/aggresome-like specific dye ProteoStat™ in pBMDM  
 891 treated with the proteasome inhibitor MG132, infected with *L. monocytogenes* or transfected with  
 892 2 μg/ml of G3-YSD for 6h. F. Quantification of the number of foci per cell containing  
 893 polyubiquitinated proteins in WT and p62<sup>-/-</sup> pBMDM treated with LPS 10 ng/ml, infected with *L.*  
 894 *monocytogenes* as above or transfected with either 2 μg/ml of G3-YSD or 25 pmol of ISD. G.  
 895 Representative images of pBMDM treated with 10 ng/ml of LPS, infected with *L. monocytogenes*,  
 896 or transfected with 2 μg/ml of G3-YSD for 6h and stained for LC3 and polyubiquitinated proteins.  
 897 Graphs represent the mean and the corresponding standard deviation of the mean from at least two  
 898 independent experiments using more than 145 cells per condition. Significant differences were  
 899 calculated using two-tailed Student's t-test (A, B, F) or one-way ANOVA with Dunnett's (D, E)  
 900 or Tukey's (G) post-tests (NS, not significant, \*\*\*\* p < 0.0001, † significantly lower). Scale bar:  
 901 10μm.

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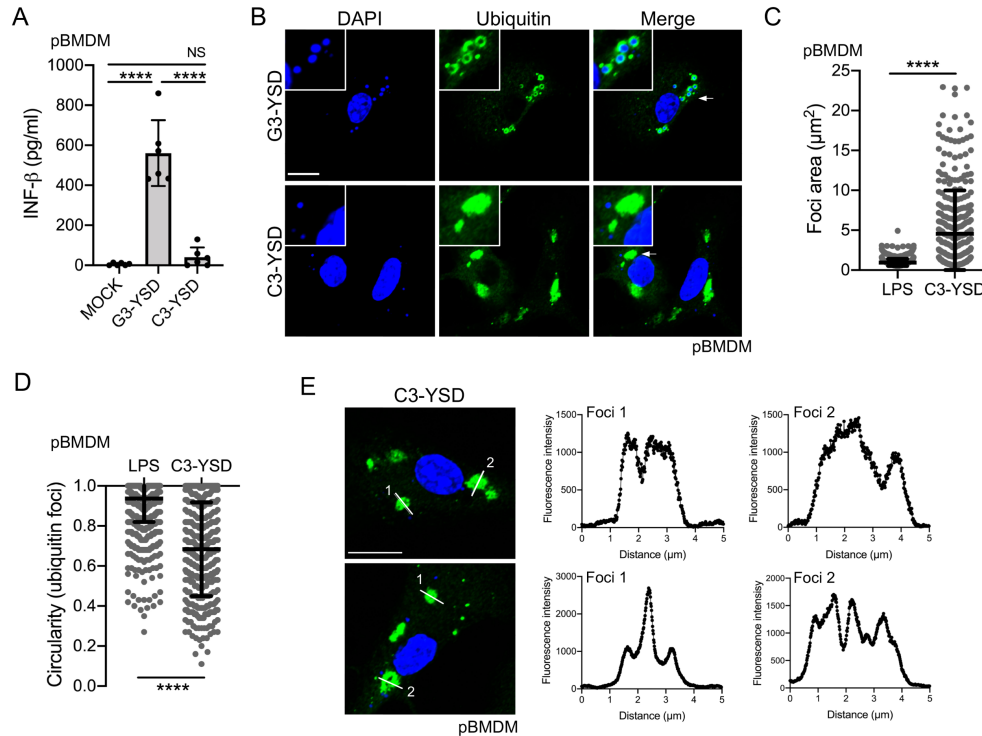
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906 **Figure 4: Proteins involved in innate immune pathways are associated with the Ub-**  
907 **containing structures.** pBMDM stimulated for 6h with 10 ng/ml of LPS or transfected with 2  
908  $\mu\text{g/ml}$  of G3-YSD were stained using antibodies against polyubiquitinated proteins and  
909 NEMO/IKK $\gamma$  (A), phosphorylated p38 MAPK (B), phosphorylated TBK1 (C) or cGAS (D).  
910 Representative images from three independent experiments are shown. The arrows indicate the  
911 regions that are displayed in higher magnification. The quantification was done according to the  
912 description in the *materials and methods* and the graphs represent the mean and the corresponding  
913 standard deviation of the mean. Significant differences were calculated using two-tailed Student's  
914 t-test (NS, not significant, \* $p < 0.05$ , \*\*\*\* $p < 0.0001$ ).

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918 **Figure 5: Functional ligand recognition is required for the formation of ubiquitin foci after**  
919 **dsDNA stimulation.** *A.* Quantification of INF- $\beta$  level in the supernatant of pBMDM transfected  
920 for 6h with 2  $\mu\text{g}/\text{ml}$  of G3-YSD or C3-YSD. *B.* Representative images of pBMDM transfected  
921 with G3-YSD and C3-YSD 2  $\mu\text{g}/\text{ml}$  for 6h and stained for polyubiquitin proteins. The transfected  
922 DNA and the nuclei were stained using DAPI. The arrows indicate the region that is displayed in  
923 higher magnification. Quantification of the foci area ( $\mu\text{m}^2$ ) (*C*) and the circularity (*D*) using the  
924 staining for polyubiquitinated proteins from pBMDM treated with 10 ng/ml of LPS or transfected  
925 with 2  $\mu\text{g}/\text{ml}$  of C3-YSD for 6h. *E.* Representative enlarged single plane confocal images showing  
926 the structures of foci stained for ubiquitinated proteins after transfection with 2  $\mu\text{g}/\text{ml}$  C3-YSD for  
927 6h. The fluorescence intensity was calculated across the white line in the neighboring panel.  
928 Significant differences were calculated using one-way ANOVA with Tukey's post-tests (*A*) or  
929 two-tailed Student's t-test (*C*, *D*) (NS, not significant, \*\*\*\*  $p < 0.0001$ ,  $\dagger$  significantly lower).  
930 Scale bar: 10 $\mu\text{m}$ .

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