1 **Stabilin-1 plays a protective role against *Listeria* monocytogenes infection**

2 **through the regulation of cytokine and chemokine production and immune cell recruitment**

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17 <u>Running tittle</u>: Stabilin-1 contributes to *Listeria* clearance

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ABSTRACT

Scavenger receptors are part of a complex surveillance system expressed by host cells to efficiently orchestrate innate immune response against bacterial infections. Stabilin-1 (STAB-1) is a scavenger receptor involved in cell trafficking, inflammation and cancer, however its role in infection remains to be elucidated. *Listeria monocytogenes* (*Lm*) is a major intracellular human food-borne pathogen causing severe infections in susceptible hosts. Using a mouse model of infection, we demonstrate here that STAB-1 controls *Lm*-induced cytokine and chemokine production and immune cell accumulation in *Lm*-infected organs. We show that STAB-1 also regulates the recruitment of myeloid cells in response to *Lm* infection and contributes to clear circulating bacteria. In addition, whereas STAB-1 appears to promote bacterial uptake by macrophages, infection by pathogenic *Listeria* induces the down regulation of STAB-1 expression and its delocalization from the host cell membrane.

We propose STAB-1 as a new SR involved in the control of *Lm* infection through the regulation of host defense mechanisms, a process that would be targeted by bacterial virulence factors to promote infection.

KEYWORDS: *Listeria*, Scavenger Receptors, STAB-1, innate immunity, infection

Introduction

Listeria monocytogenes (*Lm*) is a major human food-borne pathogen that causes listeriosis, which is highly prevalent among high-risk groups including immunocompromised people, elderly, pregnant women and neonates. Listeriosis is an overall public health concern associated with high hospitalization and mortality rates, being the most deadly food-borne infection in Europe [1]. Manifestations of the disease range from a self-limiting febrile gastroenteritis to septicaemia, meningitis and encephalitis [2]. The most severe aspects of the disease are related to the capacity of *Lm* to cross the intestinal, blood-brain and maternal-foetal barriers, evading the immune response,

58 multiplying within phagocytic and non-phagocytic cells and effectively disseminating throughout host

59 tissues [3]. These properties are shaped by an arsenal of virulence factors [4].

60 The host innate immune response is critical to elicit an early defense towards *Lm.* The containment

61 of infection requires both the participation of professional phagocytes that trap bacteria from target

62 organs, and the activation of a number of pattern recognition receptors, including scavenger

63 receptors (SRs) [5, 6]. SRs comprise a diverse and conserved family of proteins, able to bind to a

64 wide range of ligands stimulating the removal of non-self and modified-self targets [7]. They

65 contribute to maintain homeostasis and control pathogen infections, playing key functions in the

66 antimicrobial host immune response [7, 8]. The role of SRs in *Lm* infection was first revealed for SR-

67 A, SR-AI/II KO mice showing increased susceptibility to *Lm* infection and displaying increased

68 hepatic granuloma formation [9]. Later, MARCO, CD36 and SR-BI were then shown to bind *Lm* and

69 to modulate the immune response against *Lm* [10-12]. The first member of the Class H of SRs to be

70 described was STABILIN-1 (STAB-1) [13]. It is a highly conserved type I transmembrane protein

71 mainly expressed in sinusoidal endothelial cells of the spleen and liver, and on both afferent and

72 efferent arms of the lymphatic vasculature, but also in subpopulations of monocytes/macrophages,

73 and hematopoietic stem cells [13-15]. STAB-1 was implicated in lymphocyte adhesion and

74 trafficking, angiogenesis and apoptotic cell clearance, therefore being crucial to maintain tissue

75 homeostasis and resolving inflammation [16]. This SR has the ability to bind different ligands

76 including modified low-density lipoproteins [17], phosphotidylserine expressed by apoptotic cells

77 [18], secreted protein acidic and rich in cysteine (SPARC). Importantly, STAB-1 was previously found

78 to bind Gram-positive and Gram-negative bacteria *in vitro* [19]. Furthermore, it is known that this

79 receptor controls inflammatory activity, modulates T cell activation and also humoral immune

80 response [20].

81 Here we address the role of STAB-1 in host defense against *Lm* infection and investigate the impact

82 of STAB-1 deficiency on the host innate immune response against this bacterial pathogen. We reveal

83 that STAB-1 KO mice display deregulated cytokine and chemokine expression, impaired recruitment

84 of myeloid cells and increased susceptibility to *Lm* infection. In addition, whereas STAB-1 appears

85 to promote bacterial uptake by macrophages, *Lm* infection induces the down regulation of STAB-1

86 expression and its delocalization from the host cell membrane.

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Materials and Methods

Bacteria and cells

90 *Listeria monocytogenes* EGD (BUG 600) (*Lm*) and the non-pathogenic *Listeria innocua* (CLIP

91 11262) (*Li*) were grown in Brain Heart Infusion (BHI) (BD-Difco) at 37°C. *Lm* EGD transformed with

92 pNF8-GFP plasmid (*Lm* EGD$_{GFP}$) was grown in BHI supplemented with 5 µg/ml erythromycin. Human

93 acute monocytic leukemia cells, THP-1 (ATCC TIB-202), were maintained in Roswell Park Memorial

94 Institute (RPMI) 1640 medium (Lonza) supplemented with 10% foetal bovine serum (FBS)

95 (BioWest). Before bacterial infection, THP-1 cells were differentiated with 10 nM phorbol 12-

96 myristate 13-acetate for 48 h [21]. Murine macrophages J774 A.1 (ATTC TIB-67) and Raw 264.7

97 (ATTC TIB-71) were cultured in Dulbecco´s modified Eagle medium (DMEM) (Lonza), supplemented

98 with 10% FBS. Human umbilical vein endothelial cells (HUVECs) were isolated and maintained in

99 M199 culture medium supplemented with 10% FBS, heparin at 100 µg/ml and endothelial cell growth

100 supplement (ECGS) at 30 µg/ml.

101

Macrophage infection

103 Macrophages were incubated for 30 min with: 100 µg/ml of fucoidan (Sigma-Aldrich), 50 µg/ml of

104 Poly(I) or Poly(C) (Santa-Cruz-Biotechnology). Cells were infected for 30 min with exponential-phase

105 bacteria at a multiplicity of infection (MOI) of 2 and treated with 20 µg/ml of gentamicin (Lonza) for

106 60 min as described [22]. Raw macrophages were incubated with 5 µg/ml or 25 µg/ml of mouse-IgG

107 (SC-2025) or anti-STAB-1 antibody (sc-98788) 1 h before bacterial infection at MOI of 50, during 30

108 min or 20 min plus 10 min with 50 µg/ml of gentamicin. Cells were washed and lysed for CFU

109 quantification.

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Bone marrow-derived macrophages (BMDMs)

112 Mouse femurs were removed and flushed with Hank's Buffered Salt Solution (HBSS-Lonza) as

113 described [23]. Bone marrow cells were collected by centrifugation and cultured overnight in DMEM

114 supplemented with 10 mM HEPES (Gibco), 1 mM sodium pyruvate (Lonza), 10% FBS and 10%

115 L929 cell-conditioned medium (LCCM). Non-adherent cells were collected and seeded. Upon 4 days

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116   of differentiation, 10% of LCCM was added and on day 7 the medium was renewed. At day 10, latex

117   beads of 1 µm (Invitrogen) (30 min of incubation) or exponential-phase bacteria at MOI 50 (20 min

118   of infection plus 10 min with 50 µg/ml gentamicin) were added. Macrophages were washed and lysed

119   for CFU quantification or used for immunofluorescence staining.

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121   ***RNA techniques***

122   RNAs were extracted from non-infected and infected cells (TripleXtractor, GRISP), as recommended

123   by the manufacturer. Purified RNAs were reverse-transcribed (iScript, Bio-Rad-Laboratories) and

124   analysed by qPCR as described [24] or using specific primer probes (TaqMan). Gene expression

125   data were analysed by comparative Ct method [25], normalized to *HPRT1* expression. For qualitative

126   analysis, PCR was performed on cDNA (KAPA2G Mix, GRISP). Amplification products were

127   resolved in 1% (w/v) agarose gel and analysed with GelDoc XR+ System (Bio-Rad Laboratories).

128   Primers and probes are listed in Table S1.

129

130   ***Immunofluorescence***

131   *Lm* EGD$_{GFP}$ infected BMDMs were fixed in 3% paraformaldehyde (15 min), quenched with 20 mM

132   NH4Cl (1 h) and blocked with 1% BSA (sigma) in PBS (30 min). Cells were permeabilized with 0.1%

133   Triton X-100 in PBS for 5 min and labelled with Alexa Fluor 647-conjugated phalloidin (Invitrogen),

134   during 45 min on the dark. Cells were washed and slide preparations were mounted and dried at

135   room temperature. Images were captured with an Olympus BX53 fluorescence microscope. The

136   percentage of cells with intracellular bacteria or beads, and the number of intracellular bacteria or

137   beads per cell were calculated. At least 300 cells were analysed for each sample in three

138   independent experiments. Non-infected and *Lm*-infected HUVEC were incubated for 1 h with primary

139   antibody rabbit anti-STAB-1 (1:100, Millipore), diluted in 0.2% saponin (Merck) supplemented with

140   1% BSA. Cells were washed in 0.2% saponin and incubated 45 min with secondary anti-rabbit Alexa

141   488 antibody (Invitrogen). DNA was counterstained with DAPI (Sigma) and actin labelled with

142   TRITC-conjugated phalloidin. Images were collected with an Olympus BX53 fluorescence

143   microscope and processed using ImageJ.

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Cell fractionation and Immunoblotting

Cytoplasmic and membrane fractions from non-infected and *Lm*-infected cells were obtained using the Subcellular Protein Fractionation kit (Thermo Scientific). Cell samples and homogenized spleens were diluted in Laemmli buffer, resolved by SDS-PAGE on 8% gels. Samples were transferred onto nitrocellulose membrane (Bio-Rad Laboratories), blocked and blotted with rabbit anti-STAB-1 (1:500, Millipore), followed by HRP-conjugated goat anti-rabbit IgG (1:2000, P.A.R.I.S). Signals were detected using ECL (Thermo-Scientific) and digitally acquired in a ChemiDoc XRS+ system (Bio-Rad Laboratories). Signal intensity was quantified using Image J.

Cytokine ELISA

Lysis buffer 2x (200 mM Tris, 300 mM NaCl, 2% triton, pH 7.4) and Complete proteinase inhibitor (Roche) were added to homogenized organs for 30 min on ice. Supernatants were collected upon centrifugation and stored (-80ºC). Mouse serum was recovered after blood centrifugation. Cytokine production was determined using murine ELISA kit (eBioscience).

Flow cytometry

Mouse spleens were collected in ice-cold storage solution (PBS 2% FBS) and single-cell suspensions prepared using cell strainers (BD-Falcon). Cells were washed upon red blood cells lysis (150 mM NH_4Cl, 10 mM $KHCO_3$, pH 7.2 in H_2O) and cell viability was assessed by trypan blue (Life-technologies) exclusion method. Peritoneal cells were collected by washing peritoneal cavities with 5 ml of storage solution, pelleted by centrifugation, washed and cell viability was assessed. Cells were labelled with brilliant violet 510-conjugated anti-CD11b, clone M1/70; BV 421-conjugated anti-CD11c, clone N418; allophycocyanin (APC)-conjugated anti-Ly6G, clone 1A8; APC with cyanin-7 (APC/Cy7)-conjugated anti-F4/80, clone BM8; and phycoerythrin (PE)-conjugated anti-Ly6C, clone HK1.4 (BioLegend). Data were acquired in a FACS Canto II flow cytometer (BD-Biosciences) and analysed using FlowJo software (TreeStar Inc.). To determine cell numbers, event number for each cell population was normalized to the total cell number.

Animal infections

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174 STABILIN-1 full knock-out (STAB-1 KO) mice and their wild-type (WT) littermates, both with a

175 C57BL/6N, 129SvJ mixed background have been described [26]. Infections were done as described

176 [27]. Briefly, intravenous infections were performed through the tail vein with 5×10^5 colony-forming

177 units (CFUs) in PBS. Mice were euthanized 72h post-infection, spleens and livers were aseptically

178 collected and CFUs counted. Blood was recovered from mice heart. Mouse survival was assessed

179 upon intravenous infection of 10^5 CFUs. Animals were intraperitoneally injected with 10^5 CFUs (*Lm*)

180 or 5 mg/kg of LTA from *Staphylococcus aureus* (L2515 Sigma) in PBS and euthanized 6h or 24h

181 later. Animal procedures followed European Commission (directive 2010/63/EU) and Portuguese

182 (Decreto-Lei 113/2013) guidelines and were approved by the IBMC Ethics Committee and Direção

183 Geral de Veterinária (license 015301).

184

185 ***Statistics***

186 Statistics were carried out with Prism (GraphPad), using unpaired two-tailed Student's *t*-test to

187 compare means of two groups, and one-way ANOVA with Tukey's post-hoc test for pairwise

188 comparison of means from more than two groups, or with Dunnett's post-hoc test for comparison of

189 means relative to the mean of a control group.

190

191 **Results**

192 ***Scavenger Receptors are required for Lm uptake by macrophages***

193 To evaluate the overall role of SRs in *Lm* uptake by eukaryotic cells, we chemically saturated SRs

194 using different pleiotropic compounds (fucoidan, Poly (I)) known to inhibit SRs [28], before *Lm*

195 infection of human (THP-1) macrophages*.* Pre-treatment of THP-1 cells with fucoidan severely

196 impaired *Lm* uptake when compared to non-treated cells (Figure 1A). In addition, the number of

197 intracellular bacteria was also reduced upon SR saturation with Poly (I), but not with its

198 corresponding negative control Poly (C) (Figure 1A). In agreement, pre-treatment of murine

199 macrophage-like cells (Raw and J774 cell lines) with fucoidan also compromised *Lm* uptake (Figure

200 1B). These data suggested a role for SRs in *Lm* uptake by macrophages.

201 To identify SRs potentially involved in *Lm* uptake by macrophages, we assessed SR expression

202 profiles by analysing total RNAs isolated from human and murine macrophage cell lines. Our

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203 analysis revealed that, although some of the selected SRs appeared broadly expressed, each cell

204 line presented a specific SR expression profile (Figure 1C). In the tested conditions, *SR-A*, *SRCL*,

205 *SREC*, *LOX1* and *STAB-1* appeared to be expressed in all cell lines. SR-A was previously proposed

206 to play a crucial role in host defense against *Lm* infection [9]. Interestingly, STAB-1 was previously

207 implicated in lymphocyte transmigration and apoptotic cell clearance [16], and shown to bind Gram-

208 positive and Gram-negative bacteria *in vitro* [19]. Since the involvement of STAB-1 in infectious

209 processes was never assessed so far, we further explore its potential role on *Lm* infection.

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211 ***STAB-1 is required for Lm uptake by macrophages***

212 During infection, systemic bacteria are sequestered by phagocytes both in the liver and spleen [29].

213 SRs are expressed by macrophages and may function as phagocytic receptors for bacteria [19].

214 Aiming at understanding the role of STAB-1 in *Lm* uptake, we pre-incubated Raw macrophages with

215 anti-IgG (negative control) or anti-STAB-1 antibody before *Lm* infection. While the percentage of

216 adherent bacteria was similar between IgG- and anti-STAB-1 treated cells, the percentage of

217 intracellular *Lm* diminished upon macrophage treatment with anti-STAB-1 antibody (Figure 2A).

218 These data suggest that saturating STAB-1 on the surface of macrophages reduces *Lm* uptake. To

219 further address the role of STAB-1 in *Lm* uptake by macrophages, bone marrow-derived

220 macrophages (BMDMs) from both WT and STAB-1 KO mice were infected with *Lm*. As compared

221 to WT, STAB-1 KO macrophages displayed decreased numbers of intracellular *Lm* (Figure 2B).

222 Immunofluorescence quantifications of the percentage of infected cells and the number of

223 intracellular bacteria per cell confirmed the reduced capacity of STAB-1 KO macrophages to uptake

224 *Lm* (Figure 2C). STAB-1 KO macrophages also displayed a slight phagocytosis defect of non-

225 pathogenic *Listeria* (*Listeria innocua - Li*) [30], as well as of latex beads (Figure 2D). However this

226 defect appeared more pronounced for *Lm* than for *Li* or beads. STAB-1 appears thus be involved in

227 the uptake of foreign bodies by macrophages.

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229 ***STAB-1 has a protective role against Lm infection***

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230   To investigate the contribution of STAB-1 during *Lm* systemic infection *in vivo*, WT and STAB-1 KO

231   mice were intravenously infected with *Lm*. Three days later, mice were euthanized and bacterial

232   loads in spleens and livers were quantified. Bacterial numbers appeared significantly higher in the

233   organs of STAB-1 KO mice (Figure 3A), demonstrating a role for STAB-1 in the control of *Lm*

234   infection. To test if this defect in the control of infection may lead to increased mortality, mice were

235   intravenously infected with a lower dose of *Lm* and survival was monitored over time. Whereas WT

236   mice survived throughout the infection, mortality in STAB-1 KO mice reached 80% by day 18 (Figure

237   3B).

238   Altogether, our data indicate that STAB-1 promotes protection against *Lm* infection.

239

240   ***STAB-1 is required for an efficient inflammatory response and immune cell accumulation in***

241   ***Lm-infected spleens***

242   Mouse infection by *Lm* induces a robust innate inflammatory response that restricts bacterial growth

243   prior to the development of protective T cell responses. Early protective immunity against *Lm* relies

244   on the production and balance of pro-inflammatory cytokines, such as TNF-α and IL-6, and anti-

245   inflammatory cytokines, such as IL-10 [31]. To analyse the potential role for STAB-1 in the production

246   of microbicidal mediators in response to *Lm* infection, WT and STAB-1 KO mice were intravenously

247   infected with *Lm*. The production of cytokines in the spleens, livers and sera of *Lm*-infected mice

248   was evaluated by ELISA three days post-infection. As compared to WT animals, infected STAB-1

249   KO mice produced lower levels of TNF-α, IL-6 and IL-10 (Figure 4A). Importantly, this reduction of

250   the cytokine levels between WT and STAB-1 KO mice is not observed in absence of infection (Figure

251   S1). These results indicate that STAB-1 plays a role in the coordinated cytokine production elicited

252   by *Lm* infection in targeted mouse organs.

253   *Lm* entering the bloodstream are rapidly taken up by various myeloid cells in tissues. In the spleen,

254   bacteria are filtered by resident myeloid cells, including dendritic cells and professional phagocytes

255   [32]. Inflammatory stimuli also induce the recruitment of inflammatory macrophages to infected

256   tissues [33]. As STAB-1 appears to regulate the production of inflammatory cytokines in response to

257   *Lm* infection, in particular in the spleen, we hypothesized that STAB-1 could impact innate immune

258   cells recruitment to the infected spleen, a major site of bacteria replication. To test this hypothesis,

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259   WT and STAB-1 KO mice were intravenously infected with *Lm*, and three days post-infection, single-

260   cell spleen suspensions were analysed regarding myeloid cell populations by flow cytometry. As

261   compared to WT infected mice, *Lm*-infected STAB-1 KO mice showed a clear defect on myeloid

262   CD11b$^{int/hi}$ cells, which resulted from the diminished number of neutrophils (CD11b^{hi}Ly6G^{hi}) and

263   macrophages (CD11b^{hi}CD11c^{lo}) (Figure 4B). Interestingly, within the macrophage population, the

264   number of inflammatory macrophages (CD11b^{hi}Ly6C^{hi}) was also reduced in infected STAB-1 KO

265   animals (Figure 4B). In absence of infection, spleens of WT and STAB-1 KO mice showed

266   comparable myeloid cell populations (Figure S2). Taken together, these data show that STAB-1 is

267   important in controlling the recruitment of neutrophils and macrophages to the spleen of *Lm*-infected

268   mice.

269   The migration and positioning of immune cells in tissues in response to infection is mainly controlled

270   by chemokines [34]. We thus analysed the expression of neutrophil- and monocyte-attracting

271   chemokines in *Lm*-infected murine organs. In infected STAB-1 KO mouse livers, the expression of

272   all chemokines tested was decreased as compared to WT infected mice (Figure 4C, left graph). In

273   infected spleens, the expression of CCL7 and CXCL10 was also decreased in STAB-1 KO mouse

274   spleens, whereas the expression of CCL2 was increased as compared to WT (Figure 4C, right

275   graph). Differences observed between organs might be the result of niche/microenvironment

276   disparities. Altogether, these results indicate a role for STAB-1 in the recruitment of immune cells to

277   *Lm*-infection sites possibly through the expression control of attracting chemokines. These data also

278   corroborate the role of CCL7 and CXCL10 in the recruitment of inflammatory monocytes to the

279   spleen.

280

281   ***STAB-1 is important for early myeloid cells recruitment in response to Lm infection***

282   The early recruitment of immune cells to infected tissues was shown to be crucial for an effective

283   innate immune response against *Lm* [33]. To evaluate the role of STAB-1 in the early trafficking of

284   myeloid cells to the site of *Lm* infection, WT and STAB-1 KO mice were intraperitoneally infected

285   with *Lm*. Exudate cells from the peritoneal cavity were recovered 6h or 24h post-infection to evaluate

286   myeloid cell populations by flow cytometry. When compared to non-infected mice, *Lm* infection

287   appeared to trigger the recruitment of cells to the focus of infection, mainly neutrophils

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288 (CD11b^{hi}Ly6G^{hi}) and inflammatory macrophages (CD11b^{hi}Ly6C^{hi}) (Figures 5A-C). While similar cell

289 populations were detected in non-infected WT and STAB-1 KO mice (Figure 5A), we observed a

290 defect recruitment of myeloid cells in STAB-1 KO when compared to WT mice, both at 6h and 24h

291 after *Lm* infection (Figures 5B-C).

292 Lipoteichoic acids are components of Gram-positive bacteria and potent inducers of inflammation.

293 They stimulate immune cells and induce the migration of myeloid cells to the mouse abdominal cavity

294 when injected intraperitoneally [35, 36]. We used this experimental model to confirm the involvement

295 of STAB-1 in the recruitment of innate immune cells to the infection site. Purified LTA were

296 intraperitoneally injected into WT and STAB-1 KO mice and, 6h post-stimulation, exudate cells from

297 the peritoneal cavity were recovered to evaluate myeloid cell populations. In response to LTA, STAB-

298 1 KO mice showed a significant reduction in the myeloid cell (CD11b$^{int/hi}$) population when compared

299 to WT mice, which correlates to a decreased recruitment of neutrophils (CD11b^{hi}Ly6G^{hi}),

300 macrophages (CD11b^{hi}CD11c^{lo}) and inflammatory macrophages (CD11b^{hi}Ly6C^{hi}) (Figure 5D).

301 Altogether, these results indicate that, *in vivo,* STAB-1 potentiates the recruitment of immune cells

302 to the infection site upon an inflammatory stimulus.

303

304 ***STAB-1 expression is decreased and re-localized in response to Lm infection***

305 Since STAB-1 appeared to restrain *Lm* infection by regulating cytokine and chemokine production

306 and controlling myeloid cell recruitment, we investigated the potential impact of *Lm* infection on

307 STAB-1 expression. We analysed *STAB-1* expression in murine macrophage-like cells (J774) in

308 response to *Lm* infection and showed a slight decrease of *STAB-1* expression in infected as

309 compared to non-infected macrophages (Figure 6A). As observed in macrophage cell lines, *Lm*

310 infection also induced the down-regulation of STAB-1 expression in mouse BMDMs at the RNA and

311 protein level (Figure 6A and 6B). Interestingly, this down-regulation of STAB-1 expression in BMDMs

312 was only observed with pathogenic *Listeria* (*Lm*) and not with the non-pathogenic species (*Li*) (Figure

313 6A and 6B).

314 As we showed that STAB-1 is required for an efficient immune response during *Lm* infection of

315 mouse spleens, we thus assessed the impact of *Lm* infection on STAB-1 expression *in vivo,* in

316 splenic tissue. The analysis indicated that *Lm* infection also induced a large decrease of STAB-1

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317   expression in infected mouse tissues (Figure 6C). In the spleen, STAB-1 is not expressed by splenic

318   macrophages but mainly by endothelial cells [13] that were shown to be active participants in the

319   inflammatory response during *Lm* infection [37]. Therefore, we evaluated whether *Lm* infection could

320   impact STAB-1 expression on endothelial cells (HUVECs) and showed again a significant decrease

321   of STAB-1 levels in infected cells (Figure 6D). Immunofluorescence analysis corroborated the down-

322   regulation of STAB-1 expression upon *Lm* infection of endothelial cells (Figure 6E). In addition, by

323   microscopy, we also observed a de-localization of STAB-1 from the host cell membrane upon

324   infection (Figure 6E). This was further confirmed by cell fractionation that showed a significant

325   reduction of membrane STAB-1 in *Lm*-infected HUVECs when compared to non-infected cells

326   (Figure 6F).

327   Together, these results indicate that infection by pathogenic *Listeria* induces a down-regulation of

328   STAB-1 expression in infected cells and tissues. This down-regulation is accompanied by a de-

329   localization of STAB-1 from the host cell membrane.

330

331   **Discussion**

332   Scavenger receptors (SRs) are transmembrane cell surface glycoproteins restricted to

333   macrophages, dendritic cells, endothelial cells and a few other cell types [38]. Whereas SRs were

334   initially defined by their ability to bind modified low-density lipoproteins, several SRs were since

335   demonstrated to play an important role in innate immune defenses [39].

336   Here, we show for the first time the important role of the SR STAB-1 in the host protection against

337   bacterial infection. We demonstrate that, during an infection by *Listeria monocytogenes* (*Lm*), STAB-

338   1 is not only required for bacterial uptake by macrophages, but also for an efficient inflammatory

339   response, immune cell accumulation, and early myeloid cells recruitment to the infection site.

340   Interestingly, we also show that infection by pathogenic *Listeria* induces the down-regulation of

341   STAB-1 expression and its de-localization from the cell membrane, suggesting a bacterial active

342   virulence process targeting STAB-1 aiming to promote infection. STAB-1 appears thus as a new

343   important player in the host protection against a major Gram-positive food-borne pathogen.

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344   SRs were previously shown to represent an important part of the innate immune defense, in

345   particular by acting as phagocytic receptors for microorganisms [40-42]. In addition, STAB-1 was

346   previously found to bind Gram-positive and Gram-negative bioparticles *in vitro* [19], and was

347   described to be a phagocytic receptor mediating efferocytosis by recognizing phosphatidylserine on

348   apoptotic cells [43]. We show here the reduced ability of STAB-1 KO macrophages to uptake not

349   only *Lm*, but also non-pathogenic bacteria and beads. This could suggest a role for STAB-1 in the

350   general phagocytic process. However, it was previously documented that antibody blockade or

351   absence of STAB-1 is sufficient to skew macrophages from an anti-inflammatory to a more pro-

352   inflammatory phenotype [17], these later being inherently less phagocytic for *Lm* or latex beads than

353   their anti-inflammatory counterparts [44]. This could be responsible, at least in part, for the decreased

354   phagocytic capacity observed for STAB-1 KO macrophages.

355   We report here that STAB-1 contributes in the host response against *Lm* infection by controlling

356   cytokine and chemokine production, thus controlling myeloid cell recruitment. SRs are strong players

357   in the regulation of inflammation, such is the case of SR-A in *Neisseria meningitidis* and

358   *Porphyromonas gingivalis* infections [45, 46] or CD36 in response to *Staphylococcus aureus* [47].

359   STAB-1 was previously shown to control the activation of several pro-inflammatory cytokines in

360   human monocytes [48]. Here, we show that infected STAB-1 KO mice produced reduced serum,

361   liver and splenic levels of IL-6 and TNF-α as compared to WT mice, suggesting that STAB-1

362   participates in the regulation of the inflammatory cytokine response in *Lm*-targeted mouse organs

363   upon infection. In agreement, IL-6- and TNF-α-deficient mice were shown to be more susceptible to

364   *Lm* infection, with increased bacterial burden in the spleen and liver, and deficient neutrophil

365   recruitment into the blood [49, 50]. Surprisingly, we also observed an IL-10 decreased expression in

366   STAB-1 KO mice upon *Lm* infection. IL-10 is a potent inhibitor of innate immunity and IL-10 deficiency

367   was shown to improve resistance to *Lm* infection [51].

368   We show here that STAB-1 appears as an important regulator of the pro-/anti-inflammatory cytokine

369   balance in response to *Lm* infection. *Lm* uses a balance of pro- and anti-inflammatory mechanisms

370   to promote infection while inducing little inflammation in the host, both at the intestinal level and

371   systemically [52]. In a rodent model of intestinal infection, differential induction of pro- or anti-

372   inflammatory responses depending on the cell type used for entry were observed [53]. Thus a picture

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373 emerges that the modulation of pro- and anti-inflammatory properties at the cellular level has

374 important consequences for the course of infection when monitored in the complex host

375 environment. Whereas STAB-1 was previously proposed as an immunosuppressive molecule,

376 suggesting that STAB-1 may dampen pro-inflammatory reactions *in vivo* [48], our results rather tend

377 to indicate a pro-inflammatory role for STAB-1 during *Lm* infection. In the context of sepsis, where

378 the pro-inflammatory response predominates, STAB-1 was previously proposed to be both an

379 immunosuppressive player to down-regulate hyper-inflammation at early stages and to act as a

380 vascular barrier keeper in later stages of sepsis. STAB-1 could thus appear as a regulator of

381 inflammatory processes, acting both as a pro- and anti-inflammatory molecule depending of the

382 context and localization in the host.

383 Our findings also indicate that STAB-1 plays a role in the recruitment of myeloid cells in infected

384 organs, a recruitment that appears to be dependent on chemokine expression, in particular CXCL10

385 and CCL7. CXCL10 was previously involved in immune cell migration, differentiation and activation

386 [54], and is induced by TNF-α [55]. The reduced levels of CXCL10 in STAB-1 KO mice upon *Lm*

387 infection might thus correlate with the concomitant decreased expression of TNF-α. Interestingly,

388 CXCL10 has been shown to have direct antibacterial properties similar to α-defensins, in particular

389 against *Lm* [56]. During *Lm* infection, the recruitment of inflammatory macrophages from bone

390 marrow to sites of microbial infection was shown to be dependent on CCR2, a chemokine receptor

391 that responds to CCL2 and CCL7 [57]. In our experimental model, CCL7 seems to play a more

392 prominent role in the STAB-1-dependent recruitment of myeloid cells to *Lm* infected organs.

393 However, the slight increase of CCL2 expression observed in the spleen of *Lm*-infected STAB-1 KO

394 mice could also suggest a role of STAB-1 in myeloid cell chemotaxis.

395 We show that the deficiency on pro-inflammatory cytokine production and the defect on myeloid cell

396 recruitment, which are crucial for the initial control of bacterial replication, lead to higher bacterial

397 loads in the spleen and liver of STAB-1 KO mice. Neutrophils and macrophages, which are effective

398 microbicidal cells, are among the first cells involved in the *Lm*-immune response. Mice deficient for

399 these cells present increased bacterial burden and mortality [58]. The reduced capacity of STAB-1

400 KO mice to fight *Lm* infection appears thus to be more related to a deficiency in the recruitment of

401 myeloid cells to target organs, than to a killing deficiency. In agreement, STAB-1, which was shown

14

402 to be absent from all splenic macrophages, including red pulp, marginal zone and metallophilic

403 macrophages, is solely expressed by the vascular endothelium [59], and is known to be involved in

404 the transmigration of immune cells [14, 15]. Nevertheless, impaired control of the infection by STAB-

405 1 KO mice may not be only due to reduced myeloid cell recruitment to the sites of infection. STAB-

406 1 could be required for the proper migration, position and function of other immune cells, in particular

407 CD8α+ dendritic cells of the splenic marginal zone that were shown to be an obligate cellular entry

408 point for a productive infection by *Lm* [60]. However, it was previously shown that the frequencies of

409 splenic CD4+ and CD8+ T cells were comparable between wildtype and STAB-1 KO mice [20]. Our

410 work focuses on the importance of STAB-1 in the early immune response of *Lm*, but further studies

411 need to be performed to understand weather the deficiency of STAB-1 in the context of *Lm* infection

412 interferes with T cell priming and the generation of T cell memory.

413 Whereas STAB-1 appears as an important player in the host protection against *Lm*, we also show

414 that infection by this bacterial pathogen induces a decreased expression of STAB-1 in macrophages

415 and endothelial cells but also *in vivo* in infected mice spleen, which is a major target organ for *Lm*

416 replication. The expression of some other SRs, such as SR-A, MARCO and LOX-1, was also

417 reported to be modulated by microbial infection, either favoring host immune response or promoting

418 pathogen survival [45, 61, 62]. In particular, a marked expression of MARCO was observed in

419 response to *Leishmania major* and *Lm* infections [10, 63]. Interestingly, we also found that *Lm*

420 infection leads to a delocalization of STAB-1 from the membrane of endothelial cells. Importantly,

421 the down-regulation of STAB-1 expression was not observed with the nonpathogenic specie *Listeria*

422 *innocua*, that essentially differs from *Lm* by the absence of major virulence factors [30]. This suggests

423 an active mechanism driven by *Lm* virulence factors to control STAB-1 expression/localization,

424 diminish host protective responses and promote infection. However, the identification of the specific

425 virulence factors potentially involved in this process requires further investigation.

426 Here, we highlight for the first time that STAB-1 plays a protective role during *Lm* infection. By

427 regulating the inflammatory response and the recruitment of myeloid cells, STAB-1 appears as a

428 new SR with an important role for the host response against *Lm* infection. Amplifying STAB-1-

429 mediated host defenses may represent an innovative strategy against Gram-positive pathogens.

430

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Disclosure statement

The authors declare no conflict of interest.

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595
596

FIGURE LEGENDS

Figure 1. SRs are required for *Lm* uptake by macrophages. (A-B) Chemical saturation of SRs impairs bacterial uptake by macrophages-like cells. (A) Human THP-1, (B) murine Raw and J774 macrophage-like cells were left untreated or pre-treated with fucoidan, Poly(I) and its control Poly(C), infected by *Lm* for 30 min, incubated with gentamicin, washed and lysed to quantify intracellular bacteria. Values are expressed relative to values in non-treated cells, arbitrarily fixed to 100%. Values are mean ± SD of three independent assays. *$p<0.05$; **$p<0.01$; ***$p<0.001$. (C) SR gene expression was assessed by RT-PCR analysis on THP-1, Raw and J774 total RNAs, using *HPRT1* as reference gene. Band panel for each cell line is representative of two assays.

Figure 2. STAB-1 is required for *Lm* uptake by macrophages. (A) Impact of STAB-1 on *Lm* adhesion and entry into macrophages. Raw were pre-treated with an anti-IgG (SC-2025) or anti-STAB-1 antibody, at 5 µg/ml and 25 µg/ml (sc-98788) before *Lm* infection. Adherent and intracellular bacteria were quantified. Values are expressed relative to values in IgG-treated cells, arbitrarily fixed to 100%. (B) Quantification of intracellular bacteria in WT and STAB-1 KO BMDMs infected with *Lm* for 30 min. Values are expressed relative to WT arbitrarily fixed to 100%. (C) Immunofluorescence images of WT and STAB-1 KO BMDMs infected with *Lm*-GFP (green) for 30 min. Actin is labelled with TRITC-conjugated phalloidin. Scale bar, 10 µm. Quantification of the percentage of cells with intracellular *Lm* and of the number of intracellular *Lm* in BMDMs upon 30 min of infection. Values are mean ± SD of three to four independent experiments. Statistical significance is indicated as compared to WT BMDMs. *$p<0.05$; **$p<0.01$. (D) Quantification of intracellular *Li,* the percentage of cells with beads and the number of intracellular beads per cell in WT and STAB-1 KO BMDMs infected for 30 min. Values are mean ± SD of three independent experiments. Statistical significance is indicated as compared to WT BMDMs. *$p<0.05$; **$p<0.01$

Figure 3. STAB-1 have a protective role against *Lm* infection. (A) Quantification of viable bacteria in spleens and livers recovered from WT and STAB-1 KO mice, three days after intravenous infection of 5×10^5 CFU of *Lm.* Data are presented as scatter plots, each animal is represented by a dot and

21

626   the mean is indicated by a horizontal line. (B) WT and STAB-1 KO mice survival after intravenous

627   inoculation of 10^5 CFU of *Lm* (n=5). *p<0.05.

628

629   **Figure 4.** STAB-1 is required for an efficient inflammatory response and immune cell accumulation

630   in *Lm*-infected spleens. (A) WT and STAB-1 KO mice were intravenously infected with 5x10^5 CFU

631   of *Lm.* Mice were sacrificed at day three post-infection and spleen, liver and serum were collected.

632   Levels of TNF-α, IL-6 and IL-10 were measured by ELISA. Data are represented as an average of

633   ten mice from two independent experiments per group. *p<0.05; **p<0.01; ***p<0.001. (B) Spleen

634   cells from *Lm* (5x10^5 CFU) infected WT and STAB-1 KO mice were isolated and analysed by flow

635   cytometry. Total numbers of myeloid cells (CD11b$^{int/hi}$), neutrophils (CD11b^{hi}Ly6G^{hi}), dendritic cells

636   (CD11b^{int}CD11c^{hi}),   macrophages   (CD11b^{hi}CD11c^{lo})   and   inflammatory   macrophages

637   (CD11b^{hi}Ly6C^{hi}) are shown. Data are presented as scatter plots, with each animal represented by a

638   dot and the mean indicated by a horizontal line. *p<0.05; **p<0.01. (C) WT and STAB-1 KO mice

639   were intravenously infected with 5x10^5 CFU of *Lm.* Mice were sacrificed at day three post-infection

640   to recover spleens and livers. The expression of chemokines CCL2, CXCL10 and CCL7 was

641   quantified by qRT-PCR. Data are represented as an average of ten mice from two independent

642   experiments per group. *p<0.05; **p<0.01; ***p<0.001.

643

644   **Figure 5.** STAB-1 is important for early myeloid cells recruitment in response to *Lm* infection. (A-D)

645   Single-cell suspensions recovered from the peritoneal cavity of WT and STAB-1 KO mice were

646   analysed by flow cytometry to evaluate cell populations. (A) Non-infected animals. (B-C) Mice

647   intraperitoneally infected with 10^5 CFU of *Lm* for (B) 6 h or (C) 24 h. (D) Mice intraperitoneally injected

648   with purified LTA (5 mg/ml) for 6 h. Data are represented as an average of two independent

649   experiments, with at least six mice per group. *p<0.05; ***p<0.001

650

651   **Figure 6.** *STAB-1* expression is decreased and re-localized in response to *Lm* infection. (A)

652   Assessment of STAB-1 expression by (A) quantitative RT-PCR and (B) Western Blot. (A)

653   Quantification of *STAB-1* mRNA levels on RNAs extracted from J774 and BMDMs infected with *Lm*

654   for 30 min. *STAB-1* expression levels in infected conditions were normalized to those in non-infected

22

655   BMDMs, arbitrarily fixed to 1. (B-D) Independent immunoblots to detect STAB-1 protein in (B)

656   BMDMs left uninfected (NI) or infected with *Lm* or *Li* for 30 min, (C) spleen of NI and *Lm*-infected

657   mice for three days (5×10^5 CFU), (D) NI and *Lm*-infected HUVECs. Immunoblots quantification of

658   STAB-1 signal intensity in NI and infected conditions, normalized to GAPDH. (E)

659   Immunofluorescence images of NI and *Lm* infected HUVECs, stained with an anti-STAB-1 antibody.

660   DNA counterstained with DAPI and actin labelled with TRITC-conjugated phalloidin. Scale bar, 10

661   µm. (F) Immunoblots to detect STAB-1 protein in the cytoplasmic and membrane fractions of NI and

662   *Lm*-infected HUVECs upon cell fractioning. cMet was used as a membrane loading protein control

663   and GAPDH as a cytoplasmic loading protein control. Immunoblots quantification of STAB-1 signal

664   intensity in NI and infected conditions, normalized to cMet or GAPDH.

665

666   **Figure S1.** STAB-1 KO mice do not have significant defect on cytokine production. Cytokine

667   production (TNF-α, IL-6, IL-10) in spleen, liver and serum from non-infected WT and STAB-1 KO

668   mice was quantified by ELISA (n=3).

669

670   **Figure S2.** STAB-1 KO mice do not have significant defect on myeloid cell population. Spleen cells

671   from non-infected WT and STAB-1 KO mice were isolated and analysed by flow cytometry to

672   evaluate cell populations. Total numbers of myeloid cells (CD11b$^{int/hi}$), neutrophils (CD11b^{hi}Ly6G^{hi}),

673   dendritic cells (CD11b^{int}CD11c^{hi}), macrophages (CD11b^{hi}CD11c^{lo}) and inflammatory macrophages

674   (CD11b^{hi}Ly6C^{hi}) are shown. Data are presented as scatter plots, with each animal represented by a
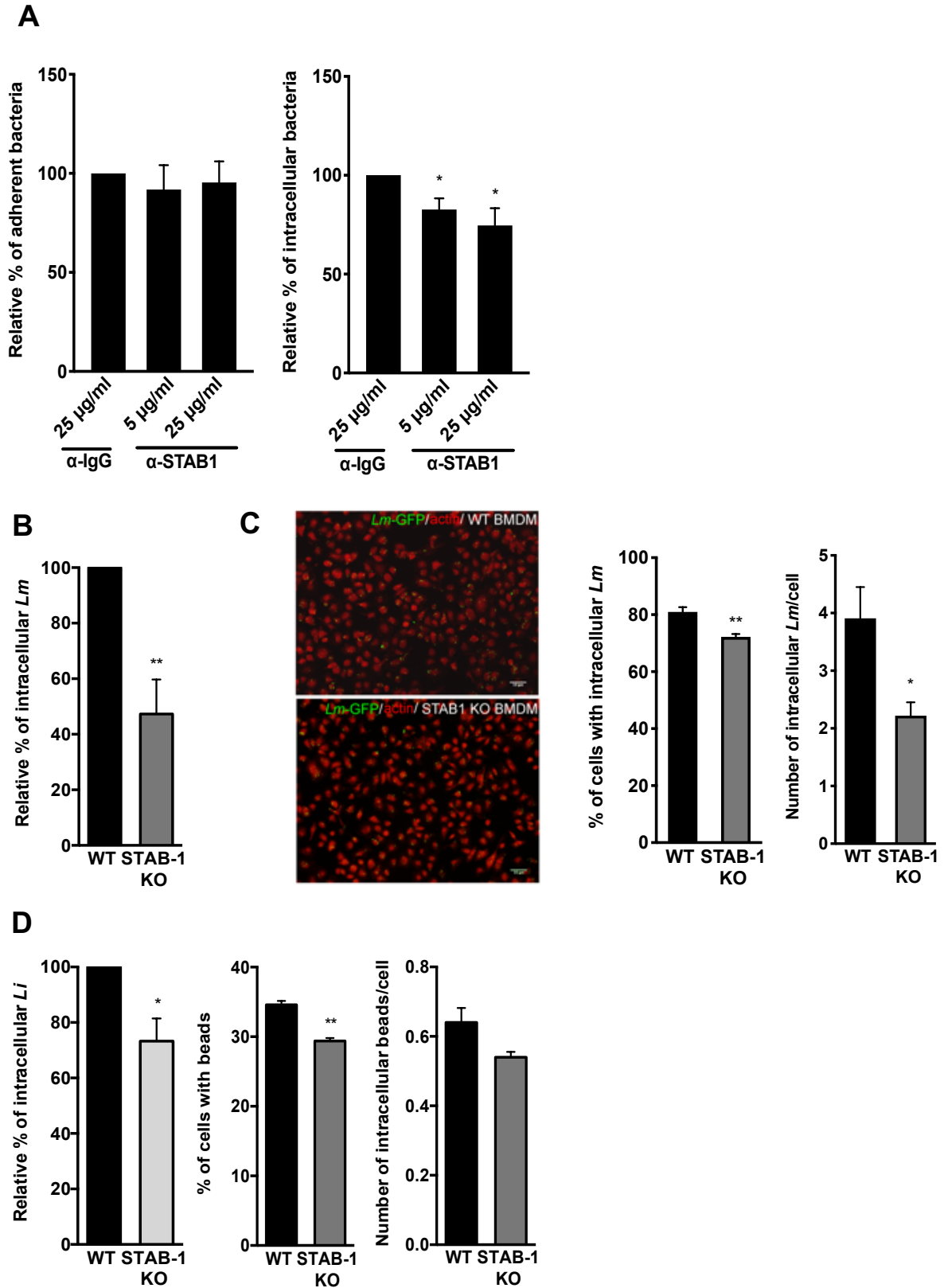
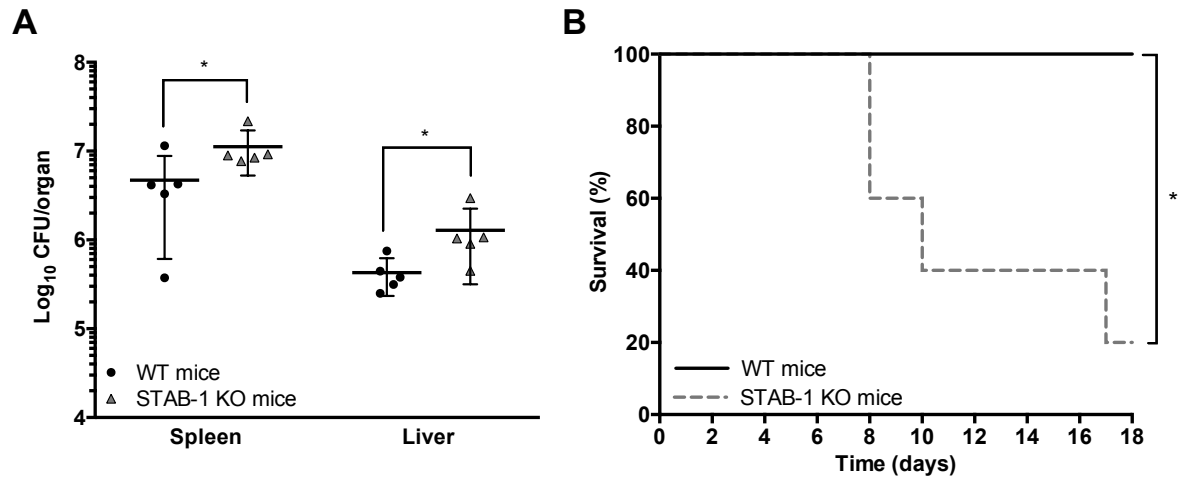675   dot and the mean indicated by a horizontal line.

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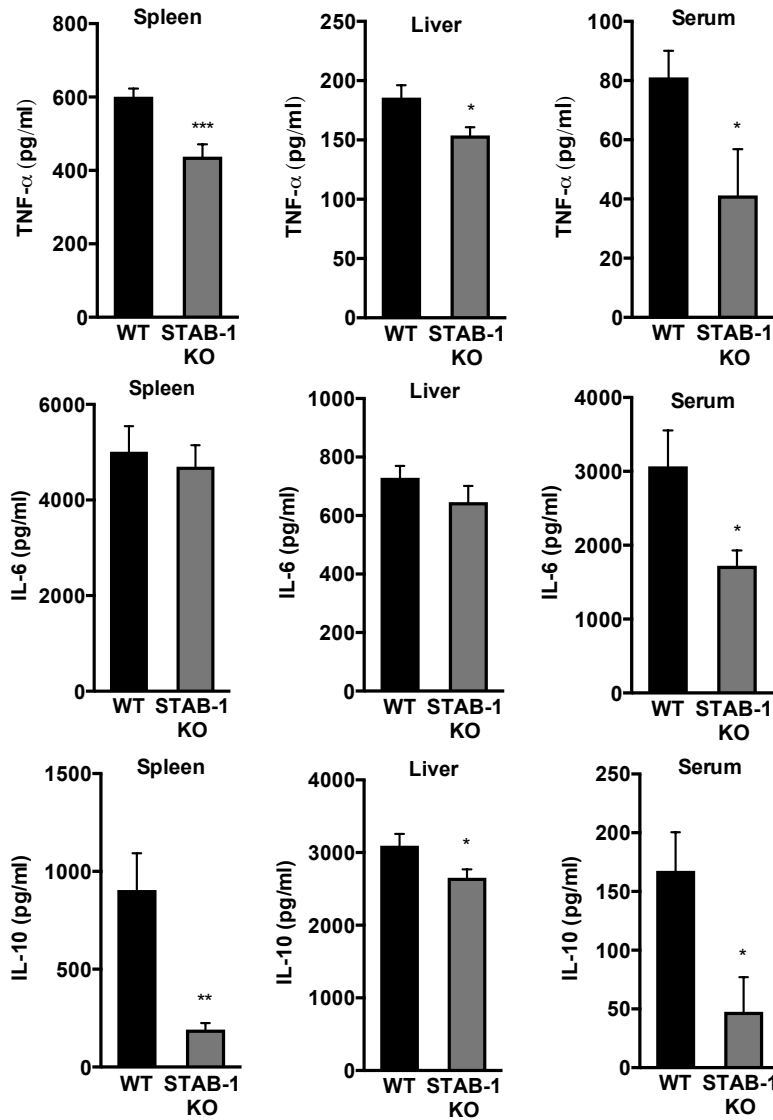**Figure 1.** SRs are required for *Lm* uptake by macrophages
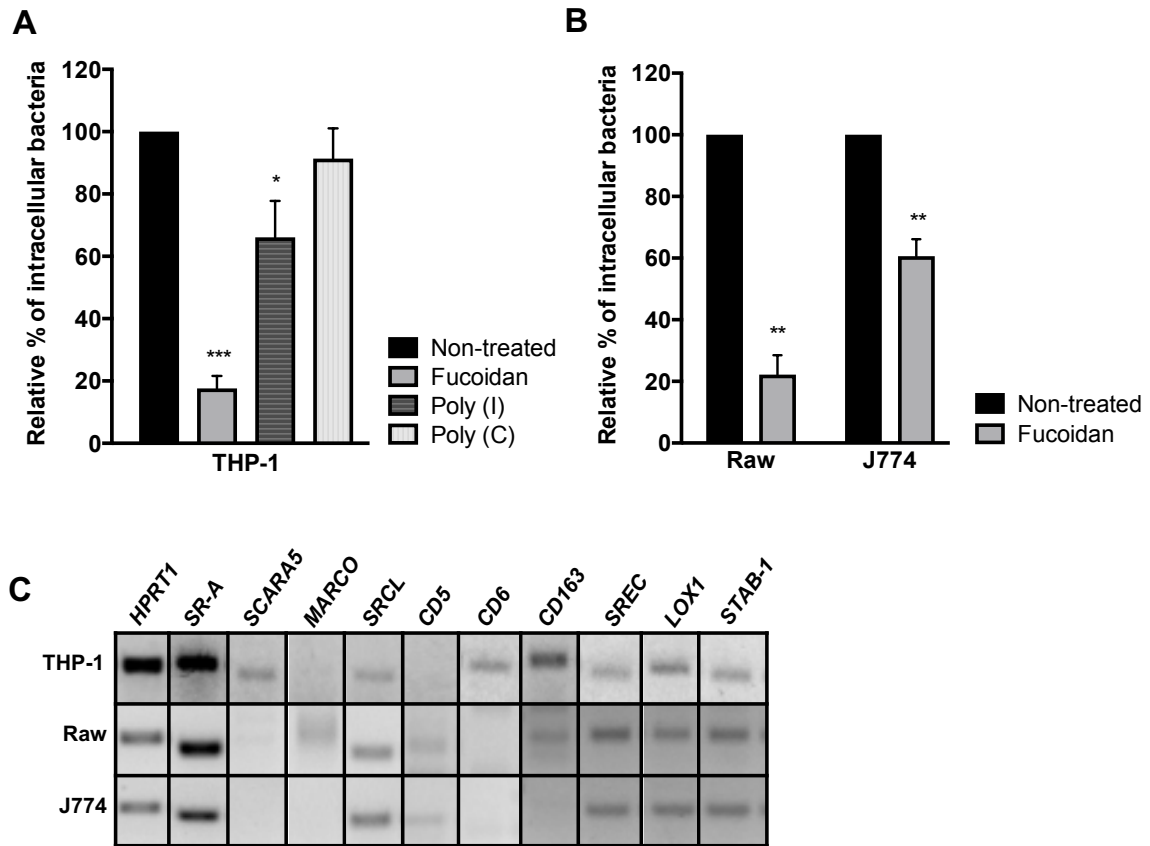
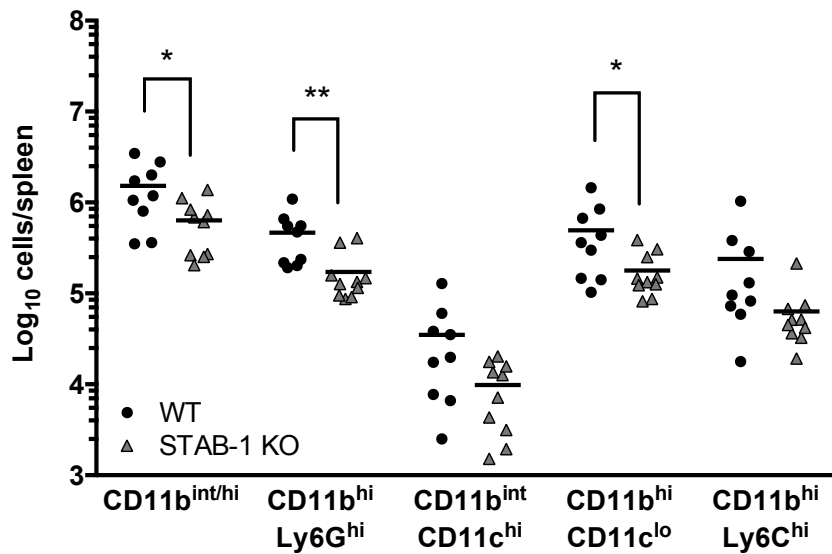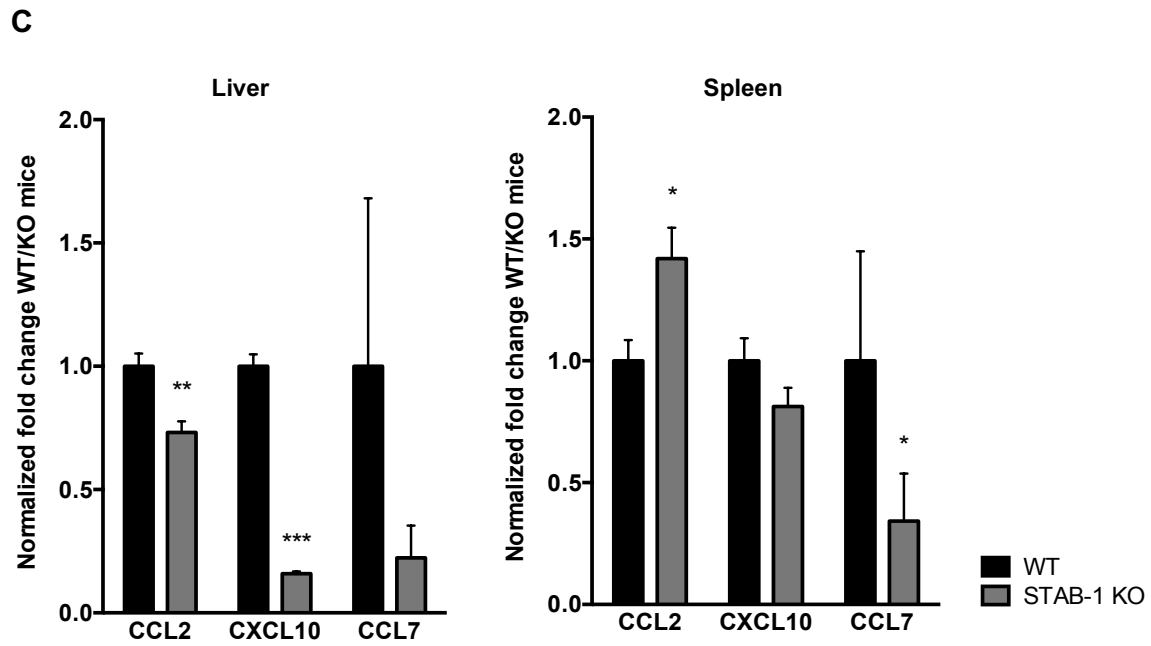**Figure 2.** STAB-1 is required for *Lm* uptake by macrophages

Figure 3. STAB-1 has a protective role against *Lm* infection
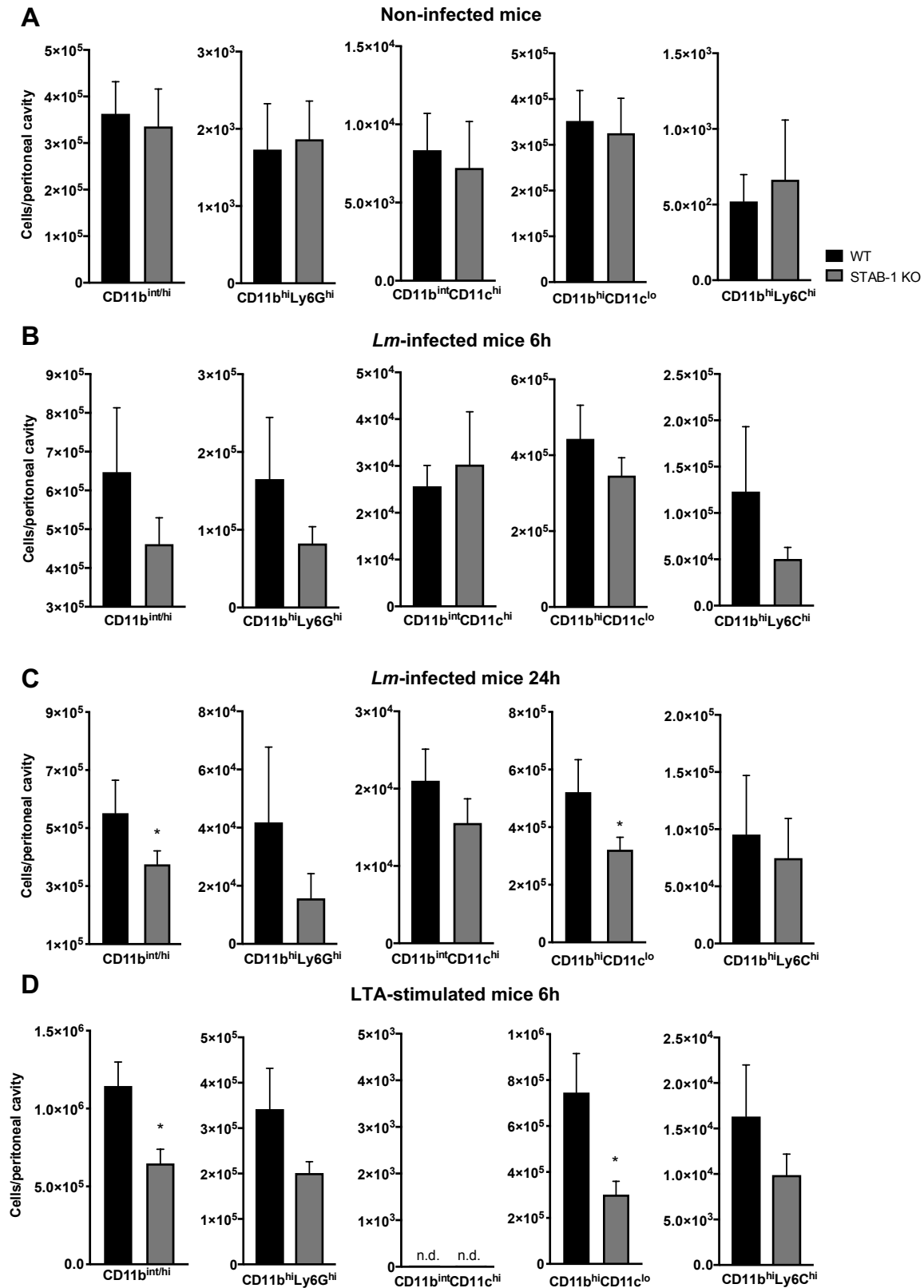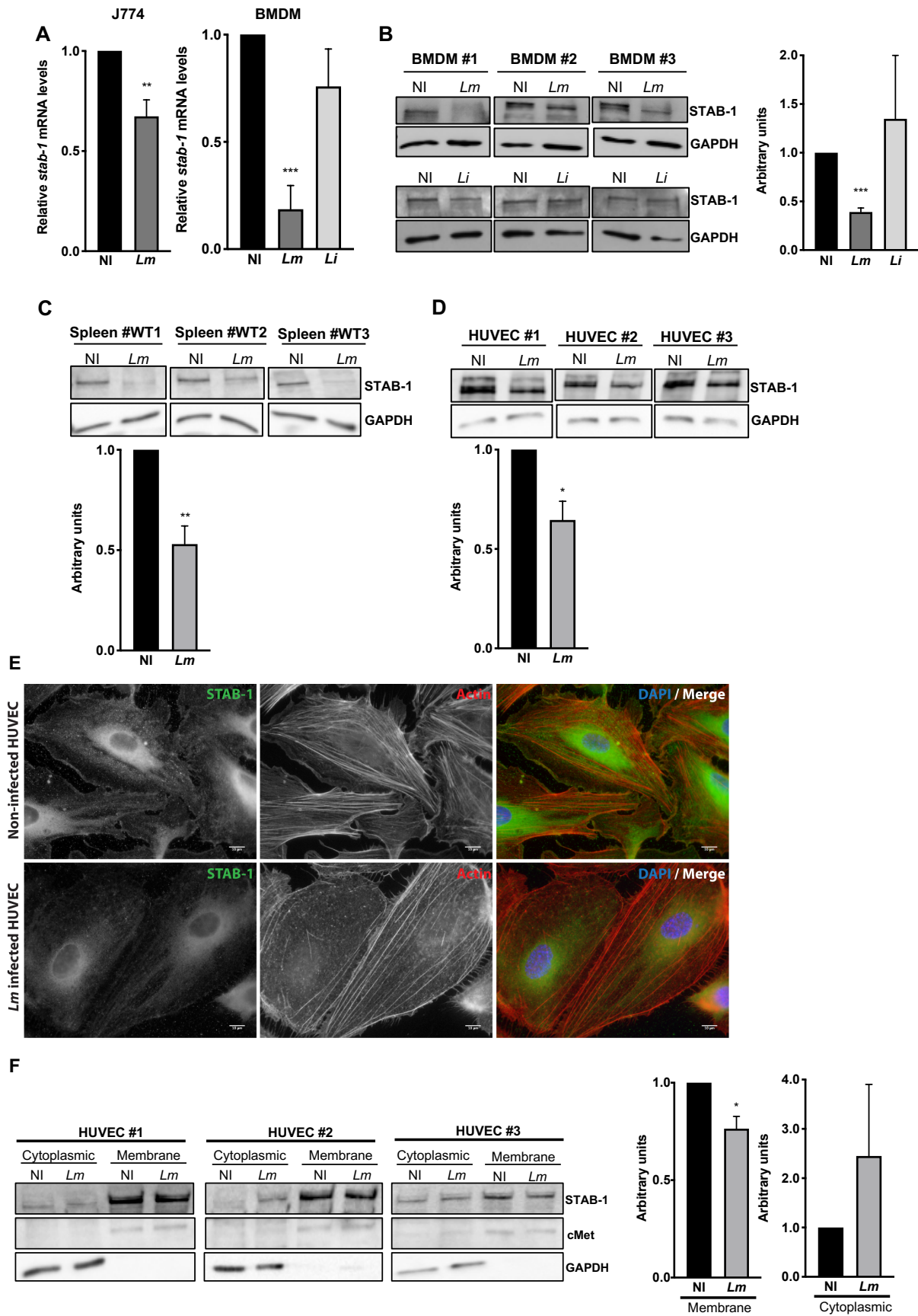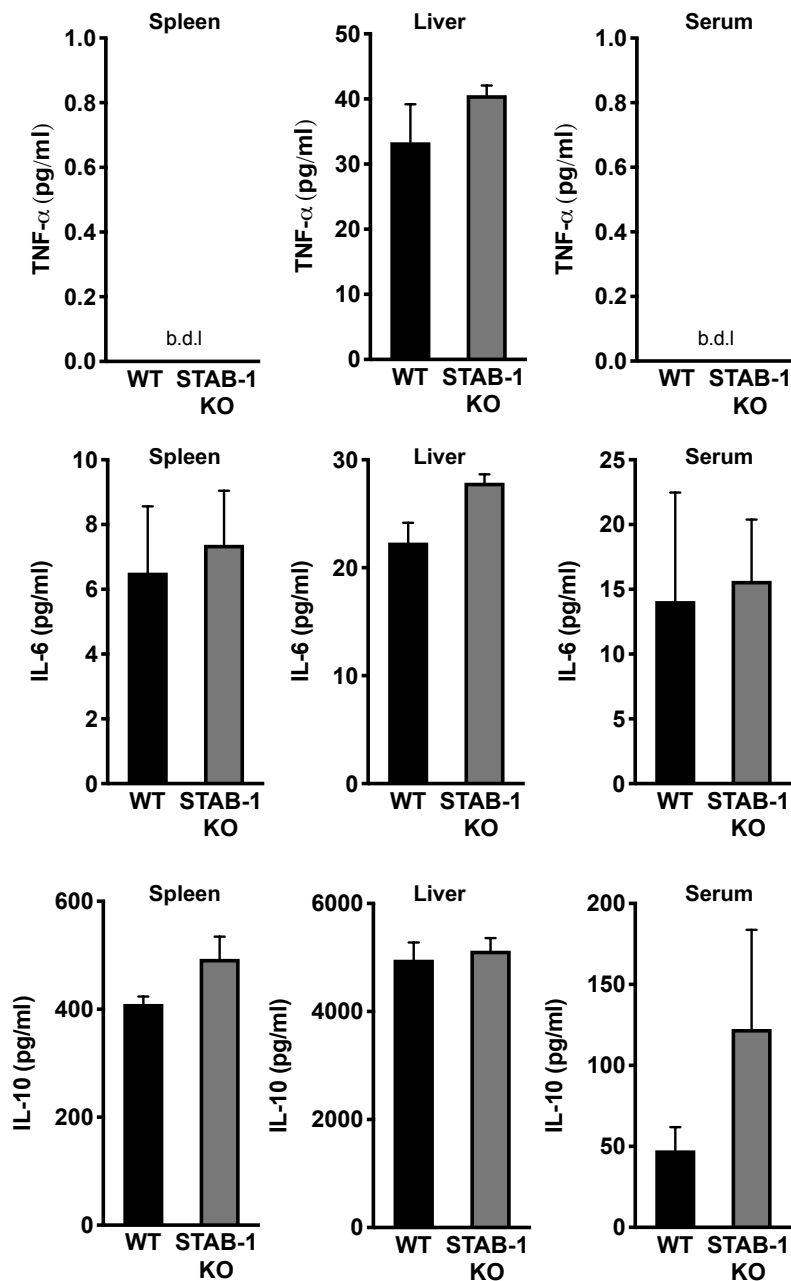
A

B

C

Figure 4. STAB-1 is required for an efficient inflammatory response and immune cell accumulation in *Lm*-infected spleens
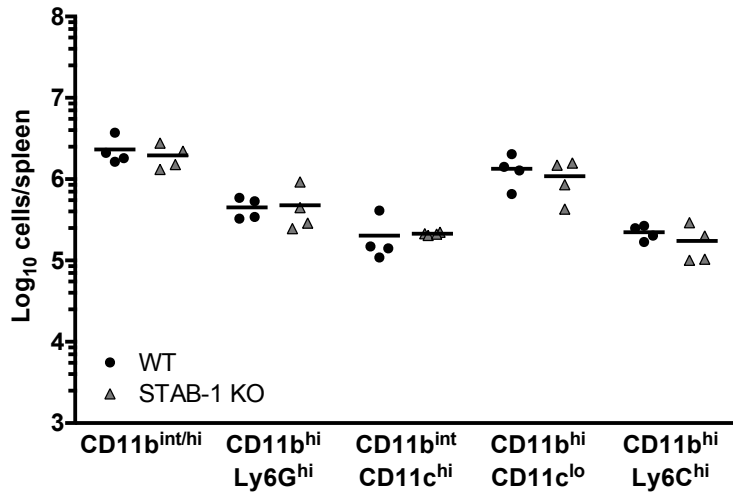
Figure 5. STAB-1 is important for early myeloid cells recruitment in response to *Lm* infection

Figure 6. *STAB-1* expression is decreased and re-localized in response to *Lm* infection

Figure S1. STAB-1 KO mice do not have significant defect on cytokine production

Figure S2. STAB-1 KO mice do not have significant defect on myeloid cell population

Table S1 Primers

Primer	Sequence (5´to 3´)	Primer	Sequence (5´to 3´)
hHPRT1 Fw	GGCGTCGTGATTAGTGATG	mHPRT1 Fw	TGATTAGCGATGATGAACCA
hHPRT1 Rv	CACCCTTTCCAAATCCTCAG	mHPRT1 Rv	GTCTTTCAGTCCTGTCCATAA
hSRA Fw	TTGAATACCACATTGCTTGATT	mSRA Fw	CTGAATATGACACTGCTTGATG
hSRA Rv	CTGATTTCCTCTTGTTGTTTGA	mSRA Rv	ATTTACTGATGTCCTCCTGTTG
hSCARA5 Fw	TTCATCTTAGCAGTGTCCAG	mSCARA5 Fw	GTGAGTGACCGTGACAAC
hSCARA5 Rv	ATTCAGCCGGTTCACATT	mSCARA5 Rv	GTGACATGGACCATCCTC
hMARCO Fw	AGAAAATTCTCAAGGAGGACGA	mMARCO Fw	GTGTGAAAGGAAGCAAGG
hMARCO Rv	TTGGGCTTTGGAACATTGATTT	mMARCO Rv	CTACAAGACCTGGGACTC
hSRCL Fw	AGTAGCCAACTTATCAGTGATTATG	mSRCL Fw	TCAGTGGTTATGGAAGAGATG
hSRCL Rv	CGGTGGACCTTGTAGTATTGTA	mSRCL Rv	CAGGAGGACCTTGTAGAATG
hCD5 Fw	TGAAGAAATTCCGCCAGAAG	mCD5 Fw	GTCTGCTTATCCAGCTCTG
hCD5 Rv	GGTTGCGATGGAAAGACAT	mCD5 Rv	AGGTCATAGTCACTGTCAG
hCD6 Fw	GTTCAGACAGTCACTATAGAATCT	mCD6 Fw	TTCCAGTCACGATAGAATCTTC
hCD6 Rv	AGGGGATGAGGAGCATTAG	mCD6 Rv	AGGACAATACAGAGAATGAGAA
hCD163 Fw	AACTTGAGTCCCTTCACCAT	mCD163 Fw	GTGTGATTTGCTTAGAGGGA
hCD163 Rv	TTGTCTGTTCCTCCAAGAGAA	mCD163 Rv	CACTTCCAATCTTCCTGAACA
hSREC Fw	ACTCCTTCTCATCCGATCC	mSREC Fw	TTCTCTTCTGATCCTGACTC
hSREC Rv	GGACCATCCCTTCTTGGG	mSREC Rv	CATAGGGACCATCTCTTCTC
hLOX1 Fw	AAAGCTAAAGGTCTTCAGTTTC	mLOX1 Fw	CCTGCTGCTATGACTCTG
hLOX1 Rv	CATAATGGTCACTACTAATCCC	mLOX1 Rv	ATACCTGGCGTAATTGTGT
hSTAB1 Fw	TAACCAATTCACGAAATACTCCTA	mSTAB1 Fw	AGGGGACTCCAAGAAAAC
hSTAB1 Rv	CCATTAGCTGCTATGTTGTTG	mSTAB1 Rv	CCACAGTTCTCCAGGATC
TNF alpha Fw	CCAAAGGGATGAGAAGTTC	**Primer-probe set reference (Applied Biosystems)**	
TNF alpha Rv	GAGAAGATGATCTGAGTGTG	HPRT1	Mm00446968_m1
IL-6 Fw	GACCTGTCTATACCACTTCAC	CCL2	Mm00441242_m1
IL-6 Rv	GCCATTGCACAACTCTTTTC	CXCL10	Mm99999072_m1
IL-10 Fw	AGCCAGGTGAAGACTTTCT	CXCL7	Mm00443113_m1
IL-10 Rv	GCAACCCAAGTAACCCTTAAAG		