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Leptospira interrogans Prevents Macrophage Cell Death and Pyroptotic IL-1 β Release through Its Atypical Lipopolysaccharide

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1 *Leptospira interrogans* prevents macrophage cell death and
2 pyroptotic IL1 β release through its atypical lipopolysaccharide

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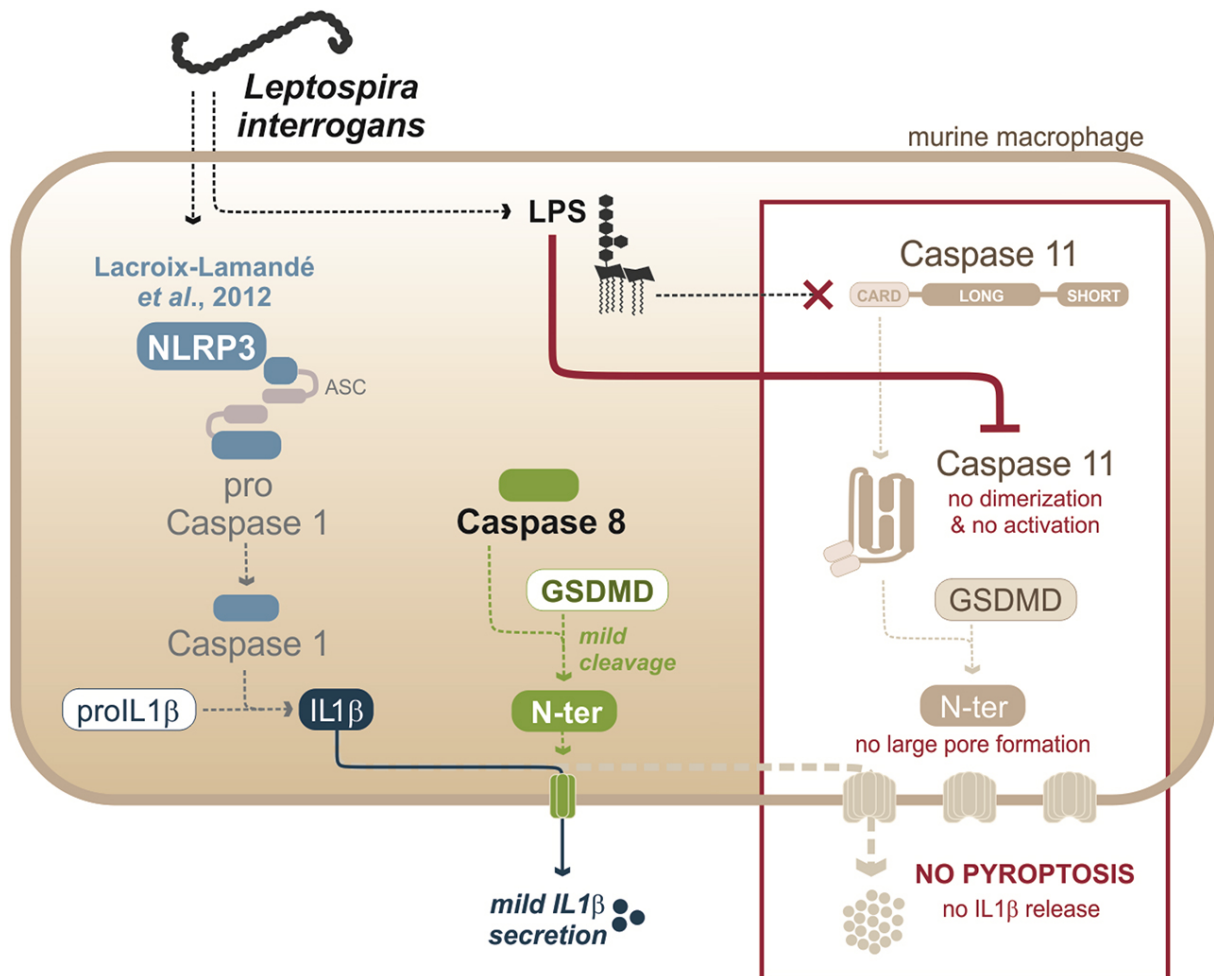
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12 **Running title**

13 Leptospire limit IL1 β secretion by pyroptosis inhibition

14
15 **Graphical abstract**



16

17 **Abstract**

18 *Leptospira interrogans* are bacteria that can infect all vertebrates and are responsible for leptospirosis,
19 a neglected zoonosis. Some hosts, such as humans, are susceptible to the disease whereas mice are
20 resistant and get chronically colonized. Although leptospires escape recognition by some immune
21 receptors, they activate the NLRP3-inflammasome and trigger IL1 β secretion. Classically, IL1 β
22 secretion is associated with lytic inflammatory cell death called pyroptosis, resulting from cytosolic LPS
23 binding to inflammatory caspases, such as caspase 11. Interestingly, we showed that *L. interrogans* and
24 *L. biflexa* do not trigger cell death in either murine, human, hamster, or bovine macrophages, escaping
25 both pyroptosis and apoptosis. We showed, in murine cells, that the mild IL1 β secretion induced by
26 leptospires occurred through non-lytic caspase 8-dependent GSDMD pore formation, and not through
27 activation of caspase 11 / non-canonical inflammasome. Strikingly, we demonstrated a potent
28 antagonistic effect of pathogenic *L. interrogans* and their atypical LPS on spontaneous and
29 *E. coli* LPS-induced cell death. Indeed, LPS of *L. interrogans* efficiently prevents caspase 11
30 dimerization and subsequent massive gasdermin D cleavage. Finally, we showed that pyroptosis escape
31 by leptospires prevents massive IL1 β release, and we consistently found no major role of IL1 receptor
32 in controlling experimental leptospirosis *in vivo*. Overall, our findings described a novel mechanism by
33 which leptospires dampen inflammation, thus potentially contributing to their stealthiness.

34

35 **Key points**

- 36 • Leptospires do not induce macrophage death, but trigger mild IL1 β secretion
- 37 • LPS from pathogenic leptospires inhibits caspase 11-induced pyroptosis
- 38 • Concealing IL1 β inside macrophages limits inflammation

39

40

41 **Introduction**

42 Leptospirosis is a re-emerging zoonosis caused by pathogenic spirochetes bacteria called
43 leptospire. Among more than 60 species, *L. interrogans* is the most prevalent and pathogenic species.
44 It can reside either free in the environment or infect vertebrates, although not all hosts show clinical
45 signs of the disease. Humans are sensitive to leptospirosis and can present symptoms that vary from a
46 mild flu-like syndrome to multi-organs failure, also known as Weil's disease (1, 2). *L. interrogans* can
47 also provoke acute symptoms in hamster, cattle, pigs and horses, such as morbidity, abortion, or
48 uveitis (1). Among the resistant animals, mice and rats get chronically colonized in the kidneys'
49 proximal tubules, leading to excretion of the bacteria in the urine, contributing to the zoonotic
50 transmission of *L. interrogans* (3, 4).

51 Upon infection, innate immunity is the first line of defense that is activated to trigger inflammatory and
52 antimicrobial responses. Microbes are recognized through conserved molecules called
53 microbe-associated molecular patterns (MAMPs) that activate host pattern-recognition receptors
54 (PRRs). Leptospire are stealth pathogens that escape some Toll-like receptors (TLR) and NOD
55 recognitions, because of particularities in their cell wall (5-7). Furthermore, unlike *Borrelia burgdorferi*
56 and *Treponema pallidum*, leptospire possess a lipopolysaccharide (LPS) in their outer membrane,
57 which is one of the few identified virulence factors (8). Its complex O antigen allows classification of
58 leptospire in more than 300 immunologically distinct serovars (3, 9). Also, the lipid A part of
59 leptospiral LPS is structurally peculiar (10) and escapes recognition by human TLR4, although it is
60 recognized by murine TLR4 (11). Interestingly, lipoproteins always co-purify with the leptospiral LPS,
61 hence conferring it the ability to activate TLR2 (12). Both the O antigen and the co-purifying
62 lipoproteins have been shown to play a role in the escape from murine TLR4-TRIF endosomal
63 signaling (6).

64 Among PRRs, activation of NOD-like receptor pyrin 3 (NLRP3) triggers the secretion of
65 interleukin-1 β (IL1 β), a tightly regulated proinflammatory cytokine, playing a central role in
66 inflammation. First, priming of NLRP3 occurs through other PRRs leading to NF- κ B translocation,
67 hence allowing NLRP3 and cytokines mRNA transcription (13, 14). NLRP3 is then activated by many
68 cellular stresses, oligomerizes and binds ASC adaptors to form a signaling platform called canonical
69 inflammasome. Inflammasomes allow cleavage of caspase 1, a protease that, in turn, cleaves pro-IL1 β
70 and pro-interleukin-18 (pro-IL18) into mature cytokines (15, 16). Interestingly, NLRP3 has also been
71 associated with the activation of caspases 4-5 (human) / caspase 11 (mouse), participating to the so-
72 called "non-canonical inflammasome" and leading to inflammatory lytic cell death with release of the
73 cytosolic content, called pyroptosis. Caspases 4-5/11 self-cleave upon intracellular binding of their
74 CARD domain to the lipid A of intracellular LPS (17) and then trigger cleavage of gasdermin D
75 (GSDMD) (17-19). Subsequently, the N-terminal fragment of GSDMD oligomerizes to form large pores

76 in the host cell membrane, leading to lytic cell death (20). NLRP3 hence contributes, through canonical
77 and non-canonical inflammasomes, to both the production and massive release of inflammatory
78 cytokines in response to infection. We have previously shown that leptospires activate the NLRP3
79 inflammasome and secretion of inflammatory IL1 β in murine macrophages, with priming through
80 TLR2/TLR4 activation by the leptospiral LPS and activation by downregulation of the
81 sodium/potassium pump by a leptospiral glycolipoprotein (21). Another study consistently reported that
82 leptospires induce NLRP3 in human cells (22, 23). Interestingly, cell death of macrophages upon
83 infection by leptospires has previously been investigated and is a matter of controversy (24).
84 Unexpectedly, we recently showed that leptospires actively enter and exit macrophages without
85 intracellular replication, and with no loss of cell viability (25). However, the potential induction of the
86 non-canonical inflammasome through activation of cytosolic caspases 4-5/11 by the leptospiral LPS had
87 never been investigated.

88 Therefore, our study aimed at characterizing potential cytotoxic effects upon infection by *L. interrogans*
89 and to assess if leptospires and their atypical LPS induce or modulate the non-canonical inflammasome
90 in addition to the canonical NLRP3 in cells from both resistant and sensitive hosts.

91 **Materials and Methods**

92 ***Leptospira* strains and cultures.** The pathogenic *L. interrogans* serovar Manilae strain L495, serovar
93 Icterohaemorrhagiae strain Verdun and serovar Copenhageni strain Fiocruz L1-130 and the saprophytic
94 *L. biflexa* serovar Patoc strain Patoc I were grown in liquid Ellinghausen-McCullough-Johnson-Harris
95 medium (EMJH) at 30 °C, without agitation. They were weekly passaged to be maintained in
96 exponential phase (twice a week in the case of *L. biflexa*). Bacteria were harvested for infection at the
97 end of the exponential phase. Bacterial concentration was adjusted by centrifuging the culture at 4000
98 g for 25 minutes at room temperature. Bacteria resuspended in PBS were counted using a Petroff-Hauser
99 chamber. Leptospire were also inactivated by heating at 56 °C for 30 min, under 300 rpm agitation.

100 **Purification of leptospiral LPS.** LPS from the different serovars of *L. interrogans* and from *L. biflexa*
101 were purified using the hot water/phenol extraction method, as we recently reviewed (26). Purified LPS
102 was used for *in vitro* cell stimulation between 100 ng/mL and 10 µg/mL.

103 **Murine macrophages culture and infection.** Bone marrow derived macrophages (BMDMs) were
104 obtained after euthanasia by cervical dislocation of adult C57BL/6J mice. Isolation of the femurs, tibia
105 and iliac bones was performed. Bone marrow cells were recovered by flushing out the bone marrow
106 using a 22 G needle in complete RPMI medium (RPMIc): RPMI (Lonza) with 10% v/v heat inactivated
107 foetal calf serum (HI-FCS, Gibco), 1 mM sodium pyruvate and 1X non-essential amino acids (Gibco)
108 supplemented with 1X penicillin-streptomycin (Gibco). Erythrocytes were lysed using red blood cells
109 lysis buffer (Sigma) and subsequent centrifugation at 1200 g for 7 minutes. Fresh or frozen bone marrow
110 cells were differentiated into macrophages by seeding 5 x 10⁶ cells in cell culture dish (TPP) with 12 mL
111 of RPMIc, supplemented with 1X penicillin-streptomycin and 10 % v/v of L929 cells supernatant.
112 Differentiation was carried out during 7 days with addition of 3 mL of medium at day 3. BMDMs were
113 recovered at day 7 by scraping in PBS-EDTA 10 mM (Gibco) and centrifuged before counting and
114 plating. Macrophage-like cell lines RAW264.7 and RAW-ASC (Invivogen), cultivated in antibiotic-free
115 RPMIc, were also used in this study. For all cells, plating was performed 24 hours before infection in
116 antibiotic-free RPMIc at a concentration of 0.3 x 10⁶ cells/mL for cell lines and 0.8 x 10⁶ cells/mL for
117 BMDMs. Cells were then infected in RPMIc with live or heat-inactivated *Leptospira* strains or
118 stimulated for 24 hours with 0.1-1 µg/mL leptospiral LPS, or with 1µg/mL *E. coli* LPS (Invivogen) and
119 2-5 mM ATP (Sigma). When indicated, RAW-ASC cells were pretreated for 1h with the following
120 caspase inhibitors: 50 µM Ac-YVAD-cmk, 20 µM Z-IETD-FMK or 10 mg/mL Z-VAD-FMK
121 (Invivogen).

122 **Human THP1-CD14 cells culture and infection.** Human THP1 monocyte-like cell line, stably
123 transfected with CD14 (originally provided by Dr. Richard Ulevitch, Scripps, San Diego, CA, USA)
124 were cultivated in antibiotic-free RPMIc. Cells were plated in RPMIc (2% v/v HI-FCS) at a

125 concentration of 0.8×10^6 cells/mL 24h before infection. Cells were then infected in antibiotic-free
126 RPMIc with live *Leptospira* strains or stimulated for 24 hours with 0.1-1 μ M nigericin.

127 **Bovine & hamster macrophages culture and infection.** Bovine bone marrow cells were obtained from
128 the femur of a neonate calf (kindly provided by Dr. Sonia Lacroix-Lamandé, INRA, Nouzilly, France).
129 Hamster bone marrow cells were obtained from femurs and tibias of golden Syrian hamsters (kindly
130 provided by Dr. Nadia Benaroudj, Institut Pasteur, Paris, France). In both cases, bone marrow cells were
131 recovered by flushing out the bone marrow using RPMIc medium (20% v/v HI-FCS). Erythrocytes were
132 lysed using red blood cells lysis buffer (Sigma) and subsequent centrifugation at 1200 g for 7 minutes.
133 Frozen bone marrow cells were thawed and differentiated into macrophages by seeding 10×10^6 cells
134 in cell culture dish (TPP) with 12 mL of RPMIc (20% v/v HI-FCS), 1X penicillin-streptomycin (Gibco)
135 and 5 ng/mL of recombinant human macrophage colony stimulating factor (hCSF, Peprotech).
136 Differentiation was carried out during 14 days with addition of 3 mL of medium every 3 days. BMDMs
137 were recovered at day 14 by scraping in PBS-EDTA 10 mM (Gibco) and centrifuged before counting.
138 Cells were plated in RPMIc medium (20% v/v HI-FCS) at a concentration of 0.8×10^6 cells/mL 24h
139 before infection. Cells were then infected in antibiotic-free RPMIc with live *Leptospira* strains or
140 stimulated for 24 hours with 1 μ g/mL *E. coli* LPS (Invivogen) and 2-5 mM ATP (Sigma).

141 **MTT viability assay.** MTT assay was performed on murine, bovine, hamster BMDMs, and on human
142 THP1-CD14 cells 24h post-infection. Briefly, all the culture supernatant was removed and a solution of
143 1 mM MTT (Sigma) in RPMIc was added on the cells. After 90 min incubation at 37°C, dissolution of
144 the formazan crystals was performed with isopropanol acidified with 1 M HCl. Optical density was
145 measured at 595 nm on a BioTek ELx800 microplate reader.

146 **Cytosolic LDH release assay.** LDH release was quantified on fresh supernatant of murine, bovine,
147 hamster BMDMs, and on RAW-ASC, RAW264.7, THP1-CD14 cells 24h post-infection or
148 post-transfection. CyQuant LDH Colorimetric & Fluorimetric assays (Invitrogen) were performed
149 according to the manufacturer's instructions. Optical density was measured at 490 nm on a BioTek
150 ELx800 microplate reader whereas fluorimetry was measured at 560 nm (*ex*) / 590 nm (*em*) on a
151 TECAN Spark fluorimeter (Life Sciences). Comparison of the kits was performed (**Sup. Figure 1B**).

152 **Caspase 3/7 activity assay.** Caspase activity was measured on murine BMDMs after overnight infection
153 or with addition of 1 μ M of staurosporine (CST) 5h prior to cell collection. Caspase 3/7 activity assay
154 (CST) was performed according to the manufacturer's instructions. Ac-DEVD-AMC substrate cleavage
155 was immediately monitored by fluorescence at 380 nm (*ex*) / 420 nm (*em*) on a TECAN Spark
156 fluorimeter (Life Sciences).

157 **Cytokine dosage by enzyme-linked immunosorbent assays (ELISA).** The secretion of cytokines
158 (murine RANTES, murine IL1 β and human IL1 β) was assessed using cell culture supernatants of
159 murine BMDMs, RAW-ASC and THP1-CD14 cells 24h post-infection. Supernatants were kept

160 at -20 °C before cytokine dosage. ELISA assays (R&D DuoSet) were performed according to the
161 manufacturer's instructions. Optical density was measured at 450 nm on a BioTek ELx800 microplate
162 reader, and cytokine concentration was determined using standard range.

163 **Nitric oxide (NO) dosage by Griess reaction.** NO dosage was performed on fresh cell culture
164 supernatant of bovine and hamster BMDMs 24h post-infection. Briefly, Griess reaction was performed
165 by incubation of 50 µL of fresh supernatant with 50 µL of 80 mM sulfanilamide in 2 M chlorohydric
166 acid. Addition of 50 µL of 4 mM N-1-naphthylethylenediamine dihydrochloride then allowed
167 colorimetric determination of NO concentration. Optical density was measured at 540 nm on a BioTek
168 ELx800 microplate reader and NO concentration was determined using standard range of nitrites.

169 **LPS transfection in RAW-ASC cells.** For LPS transfection, RAW-ASC and RAW264.7 cells were
170 plated in antibiotic-free RPMIc medium at a concentration of 0.2×10^6 cells/mL 24h before transfection,
171 in transparent bottom black plates (Greiner). Medium was removed and cells were transferred in
172 FCS-free essential medium OptiMEM (Gibco) 2h before transfection. Then, cells were transfected using
173 FUGENE (Promega) in OptiMEM (Gibco), with the equivalent of 0.5 µL/well of FUGENE, 100 ng/well
174 of DNA (either pcDNA3 or pCMV-GFP for fluorescence analyses) and LPS at a final concentration of
175 10 µg/mL. Quantities indicated correspond to the transfection of 96-well plates and were modified
176 accordingly for transfection of 24-well plates. After either 6, 12 or 24h of transfection, cells were
177 analyzed by epifluorescence microscopy, flow cytometry or fluorimetry, respectively.

178 **Propidium iodide integration analysis by flow cytometry and fluorimetry.** After either 12 or 24h of
179 transfection with LPS, RAW-ASC cells were stained with 1 mg/mL of propidium iodide (PI, Sigma).
180 For flow cytometry analyses at 12h, cells were then washed once in PBS with 2 mM EDTA and
181 0.5% v/v HI-FCS, scrapped and analyzed directly by flow cytometry on CytoFLEX (Beckman Coulter).
182 Between 10 000 and 15 000 events were acquired for each condition and the data was analyzed with
183 FlowJo V10 software. For fluorimetry analyses at 24h, the kinetics of PI integration was monitored
184 immediately at 535 nm (*ex*) / 617 nm (*em*) on a TECAN Spark fluorimeter (Life Sciences).

185 **Microscopy analyses.** Live RAW-ASC cells transfected 6h with LPS and fluorescent pCMV-GFP
186 plasmid were observed under epifluorescent inverted microscope AxioObserver Z1 (Zeiss) with 63X
187 oil-immersion objective. Brightfield illumination was maintained < 3V, laser intensity was maintained
188 <50% and exposure time was automatically set from the brightest sample (empty transfection). Images
189 analyses were performed using Fiji software. On the other hand, murine BMDMs enumeration was
190 performed after 10 min fixation with 4% w/v *para*formaldehyde in PBS and 10 min staining with 1
191 µg/mL DAPI in PBS. After three washed with PBS, fixed cells were imaged on Opera Phenix HCS
192 (Perkin Elmer) in confocal mode with 63X water-immersion objective. Laser intensity was maintained
193 <25% and exposure time was set manually to obtain arbitrary unit (AU) of fluorescence around 1 000.
194 Acquisition was performed automatically and >500 cells were analyzed *per* well. Image analysis was

195 performed automatically using Columbus software (Perkin Elmer) and the number of nuclei in each
196 condition was exported as output.

197 **SDS-PAGE and Western blots (WB).** Murine BMDMs, RAW-ASC and THP1-CD14 cells were
198 collected 8-24h post-infection or post-transfection by scrapping and centrifuged for 10 min at 1200 g.
199 Cells were then lysed in 50 μ L of RIPA lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl,
200 1% v/v Triton X-100, 0.5% w/v deoxycholate, 0.1% w/v SDS) supplemented with 1X complete Mini,
201 EDTA-free protease inhibition cocktail (Roche) for 15 min on ice. After centrifugation for 30 min at
202 full speed at 4°C, proteins were recovered in the supernatant and protein concentration was determined
203 and adjusted by Bradford dosage (BioRad). Laemmli sample buffer, supplemented with
204 10% v/v β mercapto-ethanol, was added and samples were denatured at 99 °C for 10 min. SDS-PAGE
205 of samples was performed on polyacrylamide 4-15% gradient gel in 1X Tris-Glycine-SDS buffer, at
206 110 V for 1h. Internal loading control was performed by stain free visualization after 5 min activation
207 on a ChemiDoc imaging system (BioRad), and we controlled that it was equivalent to probing the
208 membrane with anti-actin antibody (**Sup. Figure 1A**). Protein transfer was done either on nitrocellulose
209 membrane or polyvinylidene fluoride (PVDF) membrane (**Table 1**). Membranes were then saturated in
210 TBS with 0.01% v/v Tween (TBST) with 5% w/v BSA for 1h at room temperature. Incubation with
211 primary antibodies was performed in TBST with 1% w/v BSA overnight at 4°C (**Table 1**). After three
212 washes in TBST, secondary antibody incubation was done in TBST with 5% w/v BSA for 1h at room
213 temperature under mild agitation (**Table 1**). After three washes in TBST, HRP activity was revealed
214 using the SuperSignal West Femto Commercial TMB Substrate (ThermoFisher) on ChemiDoc imaging
215 system (BioRad). WB quantification was performed on raw data in ImageLab software.

216 **Native gel electrophoresis and Western blots (WB).** RAW-ASC cells were collected 12h
217 post-transfection by removal of the medium and direct lysis using commercial lysis buffer maintaining
218 caspase integrity and activity (from caspase 3/7 activity kit): 20 mM Tris-HCl pH 7.5, 150 mM NaCl,
219 1 mM Na₂ EDTA, 1 mM EGTA, 1% v/v Triton X-100, 20 mM sodium pyrophosphate, 25 mM sodium
220 fluoride, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin (CST), for 15 min on ice. After
221 centrifugation for 10 min at full speed, proteins were recovered in the supernatant and protein
222 concentration was determined and adjusted by Bradford dosage (BioRad). Native protein sample buffer
223 was then added, and electrophoresis was performed on polyacrylamide 4-15% gradient gel in
224 1X Tris-Glycine native buffer, at 90 V for 2h. Transfers and Western blots were performed as previously
225 described after denaturing SDS-PAGE.

226 **LPS-Caspase 11 binding assay.** Interaction between recombinant caspase 11 (Enzo) and *E. coli* LPS
227 (Invivogen) or *L. interrogans* LPS from serovar Manilae strain L495 was addressed by binding assays
228 performed by incubation of 100 ng of recombinant enzyme with 50-200 ng of LPS in PBS for 1h at
229 37°C. Sample were then loaded directly on home-casted native 4.5% (stacking) / 20% (running) highly

230 reticulated polyacrylamide gels, that do not allow the entrance of LPS and bound material and only
231 allow migration of unbound proteins. Electrophoresis was performed in 1X Tris-Glycine native buffer,
232 at maximum 40V for minimum 6h on ice to avoid gel heating. Transfers and Western blots were
233 performed as previously described after denaturing SDS-PAGE.

234 ***In vivo* infection with *Leptospira interrogans*.** Adult C57BL/6J mice WT were obtained from Janvier
235 Labs whereas IL1 receptor knock-out (*il1r^{-/-}*) mice were either bred at Institut Pasteur Paris animal
236 facility or provided by Bernard Ryffel (CNRS, Orléans, France). For each infection, groups of $n=3$
237 (male or female) mice were infected *via* the intra-peritoneal (IP) route with 10^8 bacteria/mouse of
238 *L. interrogans* serovar Manilae strain L495 or 2×10^8 bacteria/mouse of serovar Copenhageni strain
239 Fiocruz L1-130 in 200 μ L of endotoxin-free PBS. Naive animals were injected with 200 μ L of PBS
240 alone. Weight loss and clinical symptoms were monitored daily. At day 3 post-infection, animals were
241 euthanized by cervical dislocation and organs were recovered and frozen at -80°C . Blood and kidneys
242 were used for qPCR determination of bacterial loads and RT-qPCR analyses of cytokines mRNA.

243 **Ethic statement on animal use.** All experiments performed on animals were conducted in accordance
244 with the Animal Care guidelines and following the European Union Directive 2010/63 EU. Protocols
245 were all approved beforehand (#2013-0034 and HA-0036) by the ethic committee of the Pasteur
246 Institute, Paris, France (CETEA#89), in compliance with the French and European regulations on animal
247 welfare and according to the Public Health Service recommendations.

248 **mRNA analyses by RT-qPCR.** mRNA was extracted either from the frozen kidneys recovered 3 days
249 post-infection, or from frozen mouse BMDMs using RNeasy Mini kit (Qiagen). Reverse transcription
250 (RT) was performed using Superscript II reverse transcriptase (Invitrogen) according to the
251 manufacturer's recommendations. Generated cDNAs were used for quantitative PCR (qPCR) on a
252 StepOne Plus real-time PCR machine (Applied Biosystems), with primers and probes targeting murine
253 HPRT (internal control), caspase 11, IL1 β and IL6 (**Table 2**). The following settings were used (relative
254 quantification program): 50°C for 2 min, followed by 95°C for 10 min and by 40 cycles of 95°C for 15 s
255 and 60°C for 1 min. Data were analyzed using the comparative $2^{-\Delta\Delta\text{Ct}}$ method, using a first normalization
256 by internal control HPRT and a second normalization by non-infected controls.

257 **Bacterial loads determination by qPCR in blood.** Leptospiral DNA was extracted from blood
258 recovered 3 days post-infection using QIAmp DNA kit (Qiagen). Leptospiral DNA was specifically
259 targeted using primers and probes designed in the *lpxA* gene, conserved in *L. interrogans* strains
260 (Forward 5'-TTTTGCGTTTATTTCTGGGACTT-3' / Reverse
261 5'-CAACCATTGAGTAATCTCCGACAA-3' / Probe 5'-TGCTGTACATCAGTTTTG-3').
262 Normalization was performed using *nidogen* gene and quantitative PCR (qPCR) was performed on a
263 StepOne Plus real-time PCR machine (Applied Biosystems), with the following settings (absolute

264 quantification program): 50°C for 2 min, followed by 95°C for 10 min and by 40 cycles of 95°C for 15 s
265 and 60°C for 1 min.

266 **Statistical analyses.** All statistical analyses were performed using Student's *t*-test with corresponding
267 *p* values: * for $p < 0.05$; ** for $p < 0.01$ and *** for $p < 0.001$.

268

269 **Results**

270 **Although they induce IL1 β secretion in murine BMDMs, leptospires prevent cell death**

271 Our group previously demonstrated that infection with *L. interrogans* serovar Copenhageni strain
272 Fiocruz L1-130 activates NLRP3 inflammasome, induces caspase 1 cleavage and triggers IL1 β
273 production in murine BMDMs (21). We first assessed whether such mechanism was conserved for other
274 serovars of *L. interrogans*. BMDMs were infected for 10h with three frequently studied pathogenic
275 serovars of *L. interrogans* (serovar Manilae strain L495, serovar Icterohaemorrhagiae strain Verdun and
276 serovar Copenhageni strain Fiocruz L1-130). Western blot analyses showed that the three strains of
277 leptospires similarly induce the cleavage of caspase 1 in its active form of p10 subunit (**Figure 1A and**
278 **Sup. Figure 1A**). Consistently, all strains induced a dose-dependent secretion of IL1 β , measured 24h
279 post-infection in the supernatants of RAW-ASC cells (a murine macrophage cell line with a functional
280 inflammasome) (**Figure 1B**). Considering the activation of the canonical inflammasome by
281 *L. interrogans*, we then investigated whether leptospires also trigger the activation of the non-canonical
282 inflammasome and hence induce pyroptosis. As a marker of cell membrane damage and lytic cell death,
283 we measured the leakage of lactate dehydrogenase (LDH), a constitutive cytosolic enzyme, using either
284 colorimetric or fluorometric NADPH-dependent reactions (**Sup. Figure 1B**). Extracellular LPS from
285 *E. coli* in combination with ATP (inflammasome inducer) was used as a positive control of pyroptotic
286 cell death. Unexpectedly, although leptospires activate the canonical inflammasome, they did not induce
287 LDH release in the supernatant of BMDMs 24h post-infection (**Figure 1C**), even at high MOI,
288 suggesting that no membrane damage is occurring. Surprisingly, compared with the level of spontaneous
289 LDH released by non-stimulated cells, we even observed a reduction of the LDH released upon infection
290 with the three strains of leptospires and in the same range of magnitude at MOI 1-100. Only a reduction
291 of the infectious dose to MOI 0.1 allowed us to evidence a dose-dependent effect (**Sup. Figure 1C, left**
292 **panel**). Interestingly, such phenotype was recapitulated upon stimulation with either heat-killed
293 leptospires or purified leptospiral LPS (**Figure 1D**). Then, using MTT assay as a measure of cellular
294 metabolic activity, we confirmed that the viability of the macrophages was not reduced upon infection
295 with leptospires (**Figure 1E and Sup. Figure 1C, right panel**), and we even observed higher MTT
296 levels upon infection with MOI > 1. Similar results were obtained upon stimulation by heat-killed
297 bacteria or LPS (**Figure 1F**). All the cell death assays were performed at 24h post-infection, but we also
298 infected BMDMs with *L. interrogans* strain L495 for longer time points to exclude a delayed
299 cytotoxicity. Consistently, we observed no LDH release nor reduction in MTT 48h post-infection
300 (**Sup. Figure 1D**). Finally, to determine whether the lack of cytotoxicity was specific to the pathogenic
301 *L. interrogans*, we infected BMDMs with the saprophytic *Leptospira biflexa* serovar Patoc strain
302 Patoc I. Our results show that, like its pathogenic counterparts, *L. biflexa* induces the production of
303 IL1 β (**Sup. Figure 1E**) but does not trigger LDH release nor alter cell viability in BMDMs
304 (**Sup. Figure 1F**). Altogether, these results show that leptospires are not cytotoxic for murine BMDMs

305 and suggest that they can even prevent spontaneous cell death. Of note, LDH and MTT reflect membrane
306 integrity and metabolism, respectively, and their dosage does not exactly reflect the number of BMDMs.
307 Therefore, to conclude on a potential effect on cell death prevention, we enumerated by high-content
308 (HC) microscopy the number of BMDMs at the time of plating and 24h later, with or without leptospiral
309 infection or stimulation with their LPS. A small reduction in the number of adherent cells was observed
310 in the non-infected condition (**Figure 1G**), most likely due to basal spontaneous death. Surprisingly,
311 infection with leptospires or stimulation with their LPS efficiently prevented this spontaneous decrease,
312 which was not observed with *E. coli* LPS control stimulation (**Figure 1G**), here again showing that
313 leptospires not only are not cytotoxic, but also prevent BMDMs cell death.

314 **Leptospires do not trigger cell death in bovine and hamster BMDMs, nor in human macrophages**

315 Then, because of the ability of *L. interrogans* to infect various hosts such as humans, bovines and
316 hamsters; and because several species-specific mechanisms were discovered in the innate immune
317 escape of leptospires (7, 11), we also addressed the potential induction of pyroptosis on primary cells
318 from susceptible hosts. To do so, we derived BMDMs from bone marrow of calf and hamster and
319 infected these cells with the main three pathogenic serovars of *L. interrogans* for 24h (serovar Manilae
320 strain L495, serovar Icterohaemorrhagiae strain Verdun and serovar Copenhageni strain Fiocruz
321 L1-130). First, we observed no visible cell damage on the infected cells, unlike cells treated with LPS
322 from *E. coli* in combination with ATP (inflammasome inducer) (**Figure 2A** and **Figure 2B**,
323 *upper panels*). Viability and LDH assays further showed no cell death and no membrane alteration,
324 respectively (**Figure 2A** and **Figure 2B**, *middle panels*). Finally, as a control of infection, we measured
325 the production of nitric oxide. Results show that both bovine BMDMs and hamster BMDMs are
326 efficiently stimulated by leptospires and produce NO in response to the infection (**Figure 2A** and
327 **Figure 2B**, *lower panels*).

328 Regarding human cells, we have previously studied the infection of primary human monocytes with the
329 three different strains of *L. interrogans* and did not measure any loss of viability (Santecchia, 2019,
330 Sup Figure 1C). However, we had not measured the IL1 β secretion upon infection with leptospires.
331 Therefore, we infected human monocyte-like THP1 cells stably transfected with CD14 (hereafter called
332 THP1-CD14) with the three pathogenic serovars of *L. interrogans*. After 24h of infection, we controlled
333 that the strains induced the production of IL1 β in THP1-CD14, illustrating the activation of the
334 canonical inflammasome (**Figure 2C**, *left panel*). Consistent with the data previously obtained in
335 primary human monocytes, we did not observe LDH release nor viability decrease upon infection with
336 any of the leptospiral strains for either 24h (**Figure 2C**) or 48h (**Sup. Figure 2A**). In addition, we
337 infected THP1-CD14 cells with the saprophytic *L. biflexa* strain Patoc I. Our results show that, although
338 it induces IL1 β production (**Sup. Figure 2B**), infection with the saprophytic strain does not trigger LDH
339 release nor decrease cell viability (**Sup. Figure 2C**). Overall, these data show that the lack of cell

340 cytotoxicity upon infection with leptospires is conserved between murine, human, bovine and hamster
341 macrophages.

342 **Leptospires do not trigger the molecular pathways of apoptosis or pyroptosis in murine BMDMs**

343 Since infection with *L. interrogans* did not induce LDH release nor alter macrophage viability, we
344 specifically investigated in murine macrophages the apoptotic and pyroptotic molecular pathways by
345 studying the cleavage of caspase 3 and caspase 11 / GSDMD, respectively. Consistent with the absence
346 of cytotoxicity, leptospires did not trigger caspase 3 cleavage, after overnight infection
347 (**Figure 3A, left panel**), and no caspase 3/7 activity was measured (**Figure 3A, right panel**), in contrast
348 to staurosporine (positive control of apoptosis) added 5h prior to cell collection. These results showed
349 that leptospires do not trigger apoptosis in murine BMDMs. Interestingly, caspase 3/7 basal activity was
350 even reduced in BMDMs infection with leptospires, here again suggesting that leptospires might prevent
351 cell death. Regarding pyroptosis, Western blot analyses of caspase 11 post-infection revealed that
352 infection with *L. interrogans* serovar Manilae strain L495 induce upregulation of caspase 11, both at the
353 mRNA and protein levels, like the positive control for inflammasome (*E. coli* LPS + ATP), that induce
354 partial cell death at 2 mM and complete cell death with 5 mM ATP (**Figure 3B**). However, the
355 caspase 11 antibody did not recognize the active p25 cleaved form of the caspase, and we could therefore
356 not conclude regarding its potential cleavage. Interestingly, the parallel study of GSDMD by Western
357 blot showed that infection with leptospires only triggers minor GSDMD cleavage and does not induce
358 massive accumulation of GSDMD-N-ter, the moiety forming pores, as observed in pyroptotic cells, and
359 confirmed by quantification (**Figure 3C**). Overall, these findings show that leptospires do not trigger
360 the apoptotic and pyroptotic molecular pathways, confirming the lack of cytotoxicity observed
361 beforehand.

362 **Caspase 8 contributes to mild GSDMD cleavage and IL1 β secretion upon leptospiral infection**

363 Upon infection with leptospires, GSDMD is mildly cleaved, but does not lead to the lytic pyroptotic cell
364 death. To the extent of our knowledge, GSDMD can be cleaved either by caspase 11 or, to a lower extent
365 by caspase 1. Recent studies have also demonstrated that caspase 8 can cleave GSDMD (27-29). In
366 addition to caspase 1 activation (21), leptospires were also shown to activate caspase 8 in macrophages
367 (30). Therefore, to address the role of these different caspases in the mild GSDMD cleavage observed
368 upon infection, we infected RAW-ASC cells in presence of different caspase inhibitors and observed
369 24h post-infection the GSDMD cleavage by Western blot. The use of the caspase 8 inhibitor and of the
370 pan-caspases inhibitor, but not the caspase 1/11 inhibitor, led to a reduction of the faint GSDMD-N-ter
371 band induced upon *Leptospira* infection (**Figure 4A and Figure 4B**), showing that caspase 8
372 contributes to the mild GSDMD cleavage upon infection. After cleavage, the N-terminal fragment of
373 GSDMD classically accumulates in the cell to form small pores, and then large pores, that can lead to
374 mild IL1 β secretion and lytic cell death, respectively (31). To address whether GSDMD cleavage by

375 caspase 8 could induce small pores playing a role in IL1 β secretion, we measured the cytokine in the
376 supernatant of cells treated with the different caspase inhibitors. Inhibiting caspase 1/11 resulted in lower
377 IL1 β secretion, as expected considering that caspase 1 is cleaving the pro-IL1 β into the mature cytokine
378 (**Figure 4C**). Interestingly, we also observed a reduction in IL1 β secretion when using the caspase 8
379 inhibitor (**Figure 4C**), suggesting that GSDMD cleavage by caspase 8 contributes to the mild
380 IL1 β secretion observed upon infection. The efficiency of the caspase 8 and pan-caspases inhibitors was
381 controlled using staurosporine, an apoptosis inducer, and by monitoring in Western blot the reduction
382 in the p18 cleaved form of caspase 8 (**Figure 4D**).

383 **Transfected leptospiral LPS escapes pyroptosis and even prevents spontaneous cell death**

384 Considering that caspase 1 and caspase 11 did not contribute to the mild GSDMD cleavage observed
385 upon infection with leptospires, and considering that no cell death was observed, we hypothesized that
386 *L. interrogans* does not trigger the non-canonical inflammasome. Classically, it is the binding of
387 cytosolic *E. coli* LPS (lipid A) to caspase 11 (*via* the CARD domain) that leads to GSDMD cleavage
388 and pyroptosis. To test whether the LPS of *L. interrogans* would also fulfill this function, purified
389 leptospiral LPS was transfected into RAW-ASC cells and we monitored pyroptotic cell death. First, we
390 controlled that the LPS from *L. interrogans* and from *E. coli* were transfected with the same efficiency
391 by measuring GFP-fluorescence, produced from a plasmid co-transfected with the LPS, 1-3h
392 post-transfection (**Sup. Figure 3**). Then, we addressed 6h post transfection, by epifluorescence
393 microscopy, the morphological properties of the transfected cells, identifiable *via* their GFP
394 fluorescence. As expected, cell transfected without LPS (Empty) displayed physiological morphology,
395 whereas cell transfected with *E. coli* LPS were loosely adherent with damaged membranes as observed
396 in bright field, and with a very faint GFP intensity (**Figure 5A**). Of note, reduction of the GFP signal in
397 these cells suggests that membrane permeability was altered, allowing for the GFP signal leakage,
398 consistent with the induction of pyroptosis. In contrast, cells transfected with the leptospiral LPS showed
399 similar features as the negative control cells, suggesting that no pyroptosis had occurred (**Figure 5A**).
400 To further confirm this results, transfected cells were stained with propidium iodide (PI), a fluorescent
401 dye staining nuclei that only enters in cells upon rupture of the plasma membrane. Cells were then
402 analyzed by flow cytometry (**Figure 5B**) and fluorimetry (**Figure 5C**) at 12h and 24h post-transfection,
403 respectively. Both methods confirmed that the LPS of *L. interrogans* did not induce PI accumulation
404 even at 24h post-transfection, unlike the classical cytosolic LPS of *E. coli*, that efficiently triggered
405 pyroptosis. Interestingly, the levels of PI fluorescence were even lower upon transfection of the
406 leptospiral LPS than in the negative control cells, suggesting that the leptospiral LPS contributes to cell
407 death prevention. Furthermore, LDH release was monitored 24h post-transfection with different
408 amounts of leptospiral LPS from *L. interrogans* serovar Manilae strain L495, serovar
409 Icterohaemorrhagiae strain Verdun, serovar Copenhageni strain Fiocruz L1 130 and from *L. biflexa*
410 serovar Patoc strain Patoc I. We evidenced no LDH release upon transfection with leptospiral LPS from

411 either pathogenic *L. interrogans* or saprophytic *L. biflexa* (**Figure 5D**). Interestingly here again we
412 observed that LPS from pathogenic leptospires reduced the basal level of spontaneous LDH release of
413 murine macrophages. Finally, to confirm that the leptospiral LPS did not trigger the non-canonical
414 inflammasome, we performed native gel analysis of caspase 11 (known to dimerize upon activation)
415 after LPS transfection. Upon transfection of *E. coli* LPS, a shift of the band that could correspond to the
416 dimerized caspase 11 was visible as expected (**Figure 5E**). However, this shift was not observed upon
417 transfection of the leptospiral LPS (**Figure 5E**). Overall, these data show that transfected leptospiral
418 LPS does not induce caspase 11 dimerization and subsequent pyroptotic cell death thus escaping
419 activation of the non-canonical inflammasome.

420 **Leptospiral LPS potently inhibits *E. coli* LPS-induced cell death**

421 Our results showed that LPS from pathogenic leptospires could prevent macrophage cell death by
422 reducing spontaneous LDH release and PI accumulation upon transfection. We therefore investigated if
423 the leptospiral LPS could also protect against a strong activation of pyroptosis by transfected
424 *E. coli* LPS. To address this, we compared cell transfected with either *L. interrogans* LPS, *E. coli* LPS
425 or cells co-transfected with equal amounts of both LPS. Strikingly, the leptospiral LPS was able to
426 strongly reduce PI accumulation at both 12h and 24h post-transfection (measured by cytometry and
427 fluorimetry, respectively) (**Figure 6A, left panels**). In order to address potential competition between
428 the two toxins, we also performed sequential transfection of the two LPS and observed that the best
429 inhibition occurred upon pre-transfection of the cells with the leptospiral LPS 4h before adding the LPS
430 of *E. coli* (**Figure 6A, right panel**). Consistently, we observed that LPS from *L. interrogans* serovar
431 Manilae strain L495, serovar Icterohaemorrhagiae strain Verdun and serovar Copenhageni strain
432 Fiocruz L1-130 all decreased LDH release upon co-transfection with *E. coli* LPS, although LPS from
433 L495 was more potent (**Figure 6B, left panel**). Interestingly, we found that the LPS of the saprophytic
434 *L. biflexa* serovar Patoc strain Patoc I was not able to induce such inhibition (**Figure 6B, right panel**).
435 Furthermore, we addressed at a molecular level both the dimerization of caspase 11 and the cleavage of
436 GSDMD upon LPS co-transfection. Interestingly, the LPS of *L. interrogans* led to a reduction of both
437 caspase 11 dimerization and GSDMD cleavage induced by cytosolic LPS of *E. coli* (**Figure 6C**).
438 Classically, the mechanism by which *E. coli* LPS activates caspase 11 involves direct binding of the
439 lipid A to the CARD domain of the enzyme, hence triggering its dimerization. Therefore, we decided to
440 analyze potential binding of *L. interrogans* LPS and *E. coli* LPS to recombinant caspase 11. However,
441 we only found commercial recombinant caspase 11 enzyme that lacked the CARD binding domain
442 (**Figure 6D**). Nevertheless, we incubated this recombinant caspase 11 with the different LPS and
443 analyzed their migration on highly reticulated polyacrylamide gels (20%) that do not allow the migration
444 of the LPS / bound enzyme, but only allow migration of the unbound material. As expected, the *E. coli*
445 LPS did not bind the enzyme lacking its CARD domain. Interestingly, we observed a binding of the
446 leptospiral LPS to this recombinant caspase 11 (**Figure 6E**). Although we could not address the

447 specificity nor the nature of the interaction, this result suggests an atypical interaction between the
448 leptospiral LPS and caspase 11, that could play a role in the cell death inhibition. Finally, as we observed
449 that the leptospiral LPS was strongly inhibiting cell death by targeting caspase 11 of the non-canonical
450 inflammasome, we excluded a role for the canonical inflammasome by showing that this inhibitory
451 effect was conserved in both RAW-ASC and RAW264.7 cells that respectively harbor and lack a
452 functional canonical inflammasome (**Figure 6F**).

453 **Pyroptosis escape by leptospires prevents massive IL1 β release and IL1 receptor signaling** 454 **pathway does not contribute to the control of experimental acute leptospirosis**

455 Lastly, we investigated the functional consequences of the escape of pyroptosis by leptospires.
456 We hypothesized that, leptospiral escape of pyroptosis greatly dampens the IL1 β release induced upon
457 infection. To test such hypothesis *in vitro*, we infected RAW-ASC cells with *L. interrogans* and
458 artificially induced pyroptosis by adding ATP 3h before measuring IL1 β in cell supernatants. Results
459 showed that leptospires alone induce only mild IL1 β production 24h post infection. Interestingly, upon
460 infection followed by ATP treatment, the levels of IL1 β in the supernatant were 5 times higher
461 (**Figure 7A, left panel**). This massive release was further shown to be specific to IL1 β considering that
462 the levels of the chemokine RANTES were not modified with or without the ATP trigger of cell
463 pyroptosis (**Figure 7A, right panel**). Furthermore, considering that leptospires do escape massive IL1 β
464 secretion *in vitro*, we decided to test the importance of the IL1 β signaling pathway *in vivo* using
465 IL1 receptor (*illr*) knock-out (^{-/-}) mice. Leptospirosis is a biphasic disease that comprises an initial acute
466 phase with blood replication of leptospires, that can be lethal with the L495 strain (4). In mice infected
467 with sub-lethal doses of leptospires, the bacteria disappear from the blood stream before reappearing
468 around 8 days post-infection localized in the proximal tubules of the kidneys, where they establish a
469 life-long chronic colonization (4). We therefore performed intraperitoneal infections of both males and
470 females WT and *illr*^{-/-} mice with a lethal dose of 10⁸ *L. interrogans* serovar Manilae strain L495 *per*
471 mouse. First, we monitored the weight loss during the first 3 days of the infection, and we observed no
472 significant differences between the WT and *illr*^{-/-} (**Figure 7B, upper panel**). Consistently, we observed
473 no statistically significant differences in the survival rates of both groups (**Figure 7B, lower panel**).
474 More specifically, we also graded the symptoms of individual mice, according to a clinical chart that
475 we previously published (32). Interestingly, we observed no striking difference between the clinical
476 grades of the WT and *illr*^{-/-} mice (**Figure 7C**). At day 3 post-infection, animals of both groups had
477 reached the ethical endpoint and were consequently euthanized. We observed no difference in the
478 leptospiral loads in blood between WT and *illr*^{-/-} mice (**Figure 7D**). Finally, we measured the expression
479 levels of inflammatory cytokines in the kidneys, as a readout for inflammation in the organs targeted by
480 leptospires. We observed no differences in the expression of inflammatory markers IL6 and IL1 β
481 between WT and *illr*^{-/-} mice (**Figure 7E**). To confirm these results obtained with the lethal L495 strain,

482 we also performed acute infection with 2×10^8 *L. interrogans* serovar Copenhageni
483 strain Fiocruz L1-130 *per* mouse. Of note, this strain induces an acute but non-lethal leptospirosis. The
484 cytokine expression profiles in the kidney 3 days post-infection showed again no difference in IL6 and
485 IL1 β between WT and *illr*^{-/-} mice (**Sup. Figure 4**). In conclusion, the IL1 β signaling pathway does not
486 seem to play a major role during acute leptospirosis *in vivo*.

487

488

489 Discussion

490 Our results showed that infection with three of the main pathogenic serovars of *L. interrogans*
491 (serovars Manilae, Copenhageni and Icterohaemorrhagiae) did not induce cell death in primary murine,
492 bovine and hamster BMDMs. Also, we had previously reported no induction of cell death upon infection
493 of primary human monocytes (33) and we confirmed such findings in THP1-CD14 monocyte-like cells.
494 These findings were extended by showing a lack of cytotoxicity of the saprophytic *L. biflexa* in both
495 murine and human cells. Considering that leptospirosis is a zoonosis that affects differently all
496 vertebrates, and because the species-specificity of the innate immune response might play an essential
497 role in the course of the infection, it was our interest to address leptospiral cytotoxicity in different hosts.
498 Mice are resistant to the acute form of the disease and get chronically infected in their kidneys. On the
499 other hand, humans and bovines are susceptible and present diverse symptoms (34), and hamsters are
500 very susceptible to the disease and are the referent model for acute human leptospirosis (35). We
501 therefore showed that the lack of induced macrophage cell death upon infection with either pathogenic
502 or saprophytic leptospires is a conserved mechanism in both susceptible and resistant hosts. In murine
503 BMDMs, we further excluded induction of both pyroptosis and apoptosis, with no cleavage of GSDMD
504 and caspase 3, respectively. However, in the current literature, it has been described that *L. interrogans*
505 serovar Icterohaemorrhagiae strain Verdun induces apoptosis in Vero cells and mouse J774
506 macrophages (36) and that other serovars of *L. interrogans* could kill macrophages through either
507 necrosis (necroptosis/pyroptosis) (37) or through induction of caspase 8/3-dependent apoptosis (30). In
508 contrast, other publications using *L. interrogans* serovar Manilae and serovar Copenhageni (38) confirm
509 that infection does not have major cytotoxic effects on murine macrophages. To reconcile our results
510 with the current literature, we therefore hypothesize that different cell types (primary cells *versus* cell
511 lines; epithelial cells *versus* macrophages) could be differentially susceptible to the infection. Such
512 hypothesis is supported by a study that demonstrated differential cytotoxicity of leptospires in different
513 cell types (39). We are also aware that culture conditions of cell lines, especially over confluency at the
514 time of infection with leptospires, induce biased and aspecific cell death that could also explain the
515 discrepant results in the literature.

516 To the extent of our knowledge, *L. interrogans* would be the first bacteria to efficiently trigger
517 the canonical NLRP3 inflammasome (21), and yet escape subsequent induction of pyroptosis.
518 Furthermore, although the IL1 β production triggered by leptospires is mediated by NLRP3 and caspase
519 1, its release upon infection seems to be atypical. Indeed, our results showed that leptospires only
520 triggered mild GSDMD cleavage, independent of both caspase 1 and caspase 11, but dependent of
521 caspase 8. Several recent studies have highlighted the versatility of caspase 8, usually involved in
522 apoptosis, but also involved in numerous cell death pathways (28, 40, 41). Indeed, few studies reported
523 GSDMD cleavage and pyroptosis induction by caspase 8 (27-29). However, we do not know how
524 leptospiral-induced GSDMD cleavage is maintained at non-lytic levels. Therefore, further studies are

525 required to understand the underlying mechanism of this non-lethal role of caspase 8. We hypothesize,
526 as suggested (31), that limited accumulation of GSDMD N-ter fragments could lead to the formation
527 of small non-lytic pores, that would only allow mild secretion of IL1 β .

528 Classically, human caspases 4-5 and murine caspases 11 dimerize and are activated upon
529 binding of the lipid A moiety of the cytosolic LPS through their CARD domain (17, 42). Our results
530 showed that transfection of the leptospiral LPS did not induce the dimerization of caspase 11, unlike
531 cytosolic LPS of *E. coli*, suggesting that the activation of the enzyme did not occur. It remains to be
532 determined what leptospiral LPS feature is responsible for this phenotype. Our results showed that
533 different leptospiral serovars, defined by their different O antigen (43-45) and that harbor a conserved
534 lipid A (10, 46, 47), all escape pyroptosis induction to a similar extent, suggesting that the leptospiral
535 lipid A might be responsible for the phenotype. Although its structure is not known to this date, our
536 results also show that the LPS of *L. biflexa* is not cytotoxic. We may therefore speculate that its lipid A
537 might not be very different from the pathogenic ones. Caspases 4-5/11 all respond to both penta- and
538 hexa-acylated lipids A but tetra-acylated lipids A are not recognized by murine caspase 11 (48, 49). For
539 instance, LPS of *Francisella novicida* is tetra-acylated, and several other tetra-acylated LPS
540 (*Helicobacter pylori*, *Rhizobium galegae*) have been shown to escape caspase 11 induction (18),
541 suggesting that penta or hexa-acylation levels are prerequisite for caspase 11 activation (50, 51).
542 Considering that the leptospiral lipid A is hexa-acylated, our main hypothesis is therefore that the other
543 features of the lipid A (methylated 1-position phosphate group and lacking 4'-position phosphate group,
544 unsaturated acyl chains and amide liaisons) (10) could be responsible for lack of interaction with
545 caspase 11 and pyroptosis escape. Such hypothesis would be consistent with a study suggesting that
546 lack 4'-position phosphate group in *F. novicida* lipid A would also contribute to caspase 11 induction
547 escape (48, 50). Unfortunately, we were not able to directly assess the binding of the leptospiral lipid A
548 to the CARD domain of caspase 11, given that commercial recombinant inflammatory caspases are
549 devoid of their CARD domains, most probably because of their activation and cleavage upon binding
550 of endogenous endotoxins present during the expression process. A study consistently reported that
551 production of caspase 11 in *E. coli* is not appropriate for the study of non-activated caspase 11 (17).

552 In addition to the pyroptosis escape mechanism of the leptospiral LPS, our results strikingly
553 evidenced a cytoprotective effect of LPS from *L. interrogans* on murine macrophages. Indeed, we
554 showed a potent antagonistic effect of the leptospiral LPS on spontaneous cell death and furthermore on
555 *E. coli* LPS induced cell death. Inhibitory effects on pyroptosis induction have previously been
556 described for the atypical LPS of *Helicobacter pylori* and *Rhizobium galegae*. However, in these cases,
557 the mechanism is linked to the tetra-acylation of the lipid A (18). For *L. interrogans*, our results
558 interestingly showed that the LPS of different serovars, that have conserved lipid A (10, 46, 47), but that
559 differ in the structure of their O antigen (43-45) do not have the same ability to inhibit *E. coli* LPS
560 induced cell death. We therefore favor a role for the O antigen in the inhibitory effect of the leptospiral

561 LPS. Such hypothesis is supported by the fact that the LPS of the saprophytic *L. biflexa*, that has a very
562 short O antigen (6, 45) distinct from the pathogenic ones, is not cytoprotective. Interestingly, the
563 carbohydrate component of the leptospiral LPS is peculiar and does not have the same sugar composition
564 than the repeated units of classical LPS such as the one of *E. coli* (26, 43-45). Although the O antigen
565 section of the LPS is not supposed to be involved in caspase 11 activation, several studies previously
566 reported that it could interfere with activation of the non-canonical inflammasome. *Salmonella* and
567 *Shigella* both trigger low pyroptosis whereas mutant strains with shorter or completely
568 lacking O antigen induce higher levels of pyroptosis (52, 53). It is therefore believed that the full-length
569 O antigen could interfere with the lipid A binding to caspase 11 (53). Our results showing that the
570 leptospiral LPS binds caspase 11 through an atypical interaction, independent of the CARD domain,
571 further support the hypothesis that steric hindrance could prevent caspase 11 activation by *E. coli* LPS
572 and consequent pyroptosis induction. Overall, we believe that the escape and cytoprotective effects of
573 the leptospiral LPS would be linked to two different mechanisms, involving the lipid A and the
574 O antigen, respectively. Interestingly, our results showed that prevention of spontaneous death by the
575 leptospiral LPS is restricted to murine cells and is not visible in cells from other hosts. We may speculate
576 that the differences between the orthologs of non-canonical caspases 4-5 (present in humans & bovines)
577 and caspase 11 (present in mice), that share less than 60% amino acid homology, could account for the
578 species-specificity of the cytoprotection of the leptospiral LPS.

579 On the host side, comparing RAW264.7 and RAW-ASC cell lines, we showed that the
580 antagonistic effect of leptospiral LPS was independent of the ASC adaptor and consequently
581 independent of the canonical-inflammasome, suggesting involvement of components of the
582 non-canonical inflammasome. Our results further demonstrated that the leptospiral LPS was able to
583 prevent caspase 11 dimerization, showing that the inhibitory effect occurred upstream of the
584 caspase 11-GSDMD-pyroptosis pathway. Interestingly, other pyroptosis prevention mechanisms occur
585 downstream of caspase 11, with direct GSDMD targeting (54, 55). Overall, we propose that the
586 leptospiral LPS, through its atypical features, prevents pyroptosis very early on, which could partially
587 explain the striking efficiency of the inhibition.

588 The mechanism by which the cytoprotective leptospiral LPS is internalized in macrophages
589 remains unknown. Indeed, although some leptospires are intracellular, they do not reside nor replicate
590 inside cells (25) and their internalization is dose-dependent. However, our results showed that a
591 multiplicity of infection (MOI) of only 1 bacterium *per* macrophage, corresponding to only a few
592 macrophages with internalized leptospires, can efficiently inhibit spontaneous cell death. We therefore
593 believe that the cytoprotective effect is not restricted to *Leptospira*-infected macrophages and that other
594 LPS internalization mechanism(s) occur. As proposed for other bacteria, one hypothesis is that the LPS
595 could gain access to the cytosol through bacterial outer-membrane vesicles (OMVs) that would fuse
596 with the cell membrane (56). In addition, considering the potency of the effect (for which a dose-

597 dependency is only visible at MOI 0.1), we believe that other mechanisms, such as paracrine signaling,
598 may contribute to the retro-amplification of the cytoprotection. This is currently under investigation in
599 our group.

600 This study is consistent with previous studies from our laboratory showing that leptospire
601 already escape NOD1/2, TLR5 and TLR4-TRIF responses in murine macrophages, hence considerably
602 dampening the production of cytokines, chemokines, and antimicrobial compounds (5-7) making
603 leptospire very discrete bacteria. We had previously showed that leptospire activate NLRP3
604 inflammasome and trigger the production of IL1 β (21). Classically, the IL1 β secreted by macrophages
605 allows, once systemic, to induce fever and hepatic responses, such as C-reactive protein and complement
606 response. Furthermore, IL1 β induces potent amplification of inflammation through IL1 receptor
607 (IL1R) (57). Our results showed that pyroptosis escape upon leptospiral infection results in concealing
608 IL1 β inside macrophages. In addition, our *in vivo* results showed that the course of acute leptospirosis
609 is similar in WT and *illr*^{-/-} mice and that both genotypes express inflammatory markers at similar levels.
610 These results suggest that the mild IL1 β secretion induced upon infection is not enough to trigger a
611 massive retro-activation loop nor to mount a strong inflammatory response upon infection with
612 leptospire. Such findings are consistent with several clinical studies that showed no statistical
613 difference in IL1 β levels between control groups and patients suffering from acute leptospirosis,
614 suggesting that IL1 β is not a good marker for leptospirosis (58-60). However, we cannot exclude that
615 IL1 β and its receptor could play a role in the establishment of chronic leptospirosis, that we could not
616 investigate due to the limited available number of *illr*^{-/-} mice.

617 Overall, our study revealed a novel and unique immune escape mechanism by which leptospire
618 decorrelate the canonical and non-canonical inflammasomes, thus reducing IL1 β -mediated
619 inflammation despite NLRP3 activation.

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633

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811 **Figure legends**

812 **Figure 1. Although they induce IL1 β secretion in murine BMDMs, leptospire prevent**
813 **cell death**

814 **A)** Western Blot analysis of caspase 1 in BMDMs after 10h infection with MOI 100 of
815 *L. interrogans* serovar Manilae strain L495 (blue), serovar Icterohaemorrhagiae strain Verdun
816 (red), serovar Copenhageni strain Fiocruz L1-130 (green). **B)** IL1 β dosage by ELISA in the
817 supernatant of RAW-ASC cells after 24h infection with MOI 10-100 of the three serotypes of
818 *L. interrogans* mentioned above. **C-D)** LDH release (measured by CyQuant assay) on the
819 supernatant of BMDMs after 24h infection with **C)** MOI 1-100 of the three serotypes of
820 *L. interrogans* mentioned above, **D)** MOI 10-100 of heat-killed strain L495 or 24h stimulation
821 with 0.5-1 μ g/mL of purified leptospiral LPS from strain L495. **E-F)** Cell viability (measured
822 by MTT) assay in BMDMs after 24h infection with **E)** MOI 1-100 of the three serotypes of
823 *L. interrogans* mentioned above, **F)** MOI 10-100 of heat-killed strain L495 or 24h stimulation
824 with 0.5-1 μ g/mL of purified leptospiral LPS from L495. **C-F)** Positive control of pyroptosis
825 is 1 μ g/mL of *E. coli* LPS + 5 mM ATP. **G)** BMDMs enumeration by high-content (HC)
826 microscopy at the time of cell plating (0h), or after 24h with either infection by MOI 10-100 of
827 *L. interrogans* serovar Manilae strain L495 or stimulation with 0.1-1 μ g/mL of its purified LPS.
828 Control stimulation is performed with 1 μ g/mL of *E. coli* LPS. **A-G)** Bars correspond to mean
829 +/- SD of technical replicates ($n>4$). Data presented are representative of at least 3 independent
830 experiments.

831 **Figure 2. Leptospire do not trigger pyroptosis in bovine and hamster BMDMs, nor in**
832 **human macrophages**

833 **A-B)** Microscopy images, cell viability (measured by MTT assay), LDH release (measured by
834 CyQuant assay), and nitric oxide production (measured by Griess reaction) on **A)** bovine and
835 **B)** hamster BMDMs 24h after infection with MOI 10-100 of *L. interrogans* serovar Manilae
836 strain L495 (blue), serovar Icterohaemorrhagiae strain Verdun (red) or serovar Copenhageni
837 strain Fiocruz L1-130 (green). Positive control of pyroptosis is 1 μ g/mL of *E. coli* LPS + 5 mM
838 ATP. Bars correspond to mean +/- SD of technical replicates ($n=4$). Data presented are
839 representative of **A)** 3 independent experiments and **B)** 2 independent experiments. **C)** IL1 β
840 dosage (measured by ELISA), LDH release (measured by CyQuant assay) and cell viability
841 (measured by MTT assay) on human THP1-CD14 cells 24h after infection with MOI 10-100

842 of *L. interrogans* serovar Manilae strain L495 (blue), serovar Icterohaemorrhagiae strain
843 Verdun (red) or serovar Copenhageni strain Fiocruz L1-130 (green). Positive control of
844 pyroptosis is 1 μ M of nigericin. Bars correspond to mean \pm SD of technical replicates ($n=4$).
845 Data presented are representative of at least 3 independent experiments.

846 **Figure 3. Leptospire do not trigger apoptosis or pyroptosis molecular pathways in**
847 **murine BMDMs**

848 **A) Left panel.** Western blot analysis of caspase 3 in BMDMs after overnight infection with
849 MOI 10-100 of *L. interrogans* serovar Manilae strain L495 or with addition of 1-5 μ M of
850 staurosporine 5h *prior* to cell collection as positive control for apoptosis induction. *Right panel.*
851 Kinetics of caspase 3/7 activity, assessed by fluorometry measures every 15 min of cleaved
852 substrate Ac-DEVD-AMC, in BMDMs after overnight infection with MOI 100 of
853 *L. interrogans* serovar Manilae strain L495, or control stimulation with addition of 1 μ M of
854 staurosporine 5h *prior* to cell collection as positive control for apoptosis induction. Dots
855 correspond to mean \pm SD of technical replicates ($n=6$). **B)** Western blot and mRNA RT-qPCR
856 analyses of caspase 11 in BMDMs after either 8h (WB, *left panel*) or 6h (qPCR, *right panel*)
857 infection with MOI 100 of *L. interrogans* serovar Manilae strain L495 or positive control of
858 pyroptosis 1 μ g/mL of *E. coli* LPS + 2-5 mM ATP. **C)** Western blot analysis (*left panel*) and
859 quantification (*right panel*) in BMDMs and RAW-ASC cells after 8h infection with MOI 100
860 of *L. interrogans* serovar Manilae strain L495 or positive control of pyroptosis 1 μ g/mL of *E.*
861 *coli* LPS + 2-5 mM ATP. **B-C)** Bars correspond to the mean of at least 3 independent
862 experiments. **A-C)** Data presented are representative of at least 3 independent experiments.

863 **Figure 4. Caspase 8 contributes to mild GSDMD cleavage and IL1 β secretion upon**
864 **leptospiral infection**

865 **A)** Western blot analysis, **B)** WB quantification of GSDMD in RAW-ASC cells and **C)** IL1 β
866 dosage (measured by ELISA) in the supernatant of cells pretreated for 30 min with caspase 1/11
867 inhibitor (Ac-YVAD-cmk, 50 μ M), caspase 8 inhibitor (Z-IETD-FMK, 20 μ M) or
868 pan-caspases inhibitor (Z-VAD-FMK, 10 μ g/mL) and then infected for 16h with MOI 100 of
869 *L. interrogans* serovar Manilae strain L495. Bars correspond to mean \pm SD of technical
870 replicates ($n=3$). **D)** Western blot analysis of caspase 8 in RAW-ASC cells pretreated with
871 caspase 8 inhibitor (Z-IETD-FMK, 20 μ M) or pan-caspase inhibitor (Z-VAD-FMK,

872 10 µg/mL) and stimulated for 5h with 0.5-2 µM of staurosporine. **A-C)** Data presented are
873 representative of at least 3 independent experiments.

874 **Figure 5. Transfected leptospiral LPS escapes pyroptosis and even prevents spontaneous**
875 **cell death**

876 **A)** Epifluorescence microscopy of RAW-ASC cells after 6h transfection with pCMV-GFP and
877 either 10 µg/mL *E. coli* LPS or 10 µg/mL *L. interrogans* LPS from serovar Manilae strain
878 L495. **B)** Cell death monitored by flow cytometry analysis of propidium iodide (PI) integration
879 in RAW-ASC cells after 12h transfection with pCMV-GFP and either 10 µg/mL *E. coli* LPS or
880 10 µg/mL *L. interrogans* LPS from serovar Manilae strain L495. **C)** Cell death monitored by
881 fluorimetry analysis of PI integration in RAW-ASC cells after 24h transfection with pcDNA3
882 and either 10 µg/mL *E. coli* LPS or 10 µg/mL *L. interrogans* LPS from serovar Manilae strain
883 L495. Lines correspond to mean +/- SD of technical replicates (*n*=4). **D)** LDH release
884 (measured by CyQuant assay) on the supernatants of RAW-ASC cells after 24h transfection
885 with pcDNA3 and either 10 µg/mL *E. coli* LPS or 10 µg/mL *L. interrogans* LPS from serovar
886 Manilae strain L495 (blue), serovar Icterohaemorrhagiae strain Verdun (red), serovar
887 Copenhageni strain Fiocruz L1-130 (green) or *L. biflexa* LPS from serovar Patoc strain Patoc I
888 (orange). Bars correspond to mean +/- SD of technical replicates (*n*=6). **E)** Western blot
889 analysis in native conditions of caspase 11 in RAW-ASC cells after 24h transfection with
890 pCMV-GFP and either 10 µg/mL *E. coli* LPS or 10 µg/mL *L. interrogans* LPS from serovar
891 Manilae strain L495. **A-E)** Data presented are representative of at least 3 independent
892 experiments.

893 **Figure 6. Leptospiral LPS potently inhibits *E. coli* LPS-induced cell death**

894 **A)** Cell death monitored by analysis of propidium iodide (PI) integration by flow cytometry
895 12h post-transfection and by fluorimetry 24h post-transfection in RAW-ASC cells, after
896 transfection with pCMV-GFP and 10 µg/mL *E. coli* LPS, 10 µg/mL *L. interrogans* LPS from
897 serovar Manilae strain L495, or after either co-transfection or sequential transfection of
898 10 µg/mL of both LPS. **B)** LDH release (measured by CyQuant assay) on the supernatant of
899 RAW-ASC cells after 24h transfection with pcDNA3 and 10 µg/mL *E. coli* LPS + 10 µg/mL
900 *L. interrogans* LPS from serovar Manilae strain L495 (blue), serovar Icterohaemorrhagiae
901 strain Verdun (red), serovar Copenhageni strain Fiocruz L1-130 (green) or *L. biflexa* LPS from
902 serovar Patoc strain Patoc 1 (orange). Bars correspond to mean +/- SD of technical replicates

903 ($n=6$). **C) Left panel.** Western Blot analysis in native conditions of caspase 11 in RAW-ASC
904 cells after 12h transfection with pCMV-GFP and 10 $\mu\text{g}/\text{mL}$ *E. coli* LPS, 10 $\mu\text{g}/\text{mL}$
905 *L. interrogans* LPS from serovar Manilae strain L495 or 10 $\mu\text{g}/\text{mL}$ of both LPS.
906 **C) Right panel.** Western Blot analysis of GSDMD in RAW-ASC cells after 12h transfection
907 with pCMV-GFP and 10 $\mu\text{g}/\text{mL}$ *E. coli* LPS, 10 $\mu\text{g}/\text{mL}$ *L. interrogans* LPS from serovar
908 Manilae strain L495 or 10 $\mu\text{g}/\text{mL}$ of both LPS. **D)** Schematic representation of full length and
909 recombinant caspase 11 lacking the LPS-binding CARD domain. **E)** Binding assay between
910 recombinant caspase 11 and *E. coli* LPS or *L. interrogans* LPS from serovar Manilae strain
911 L495, performed on highly reticulated 20% polyacrylamide native gels that do not allow the
912 entrance of LPS and bound material and only allow migration of unbound proteins. **F)** LDH
913 release (measured by CyQuant) assay on the supernatant of either RAW-ASC or parental
914 RAW264.7 cells 24h after transfection with pcDNA3 and 10 $\mu\text{g}/\text{mL}$ *E. coli* LPS, 10 $\mu\text{g}/\text{mL}$
915 *L. interrogans* LPS from serovar Manilae strain L495 or 10 $\mu\text{g}/\text{mL}$ of both LPS. Bars
916 correspond to mean \pm SD of technical replicates ($n=3$). **A-E)** Data presented are representative
917 of at least 3 independent experiments.

918 **Figure 7. Pyroptosis escape by leptospire prevents massive IL1 β release and IL1R**
919 **signaling pathway does not contribute to the control of experimental leptospirosis**

920 **A)** IL1 β and RANTES dosage by ELISA in the supernatant of RAW-ASC cells 24h after
921 infection with MOI 100 of *L. interrogans* serovar Manilae strain L495, with or without addition
922 of 5 mM ATP 3h before supernatant collection. Bars correspond to mean \pm SD of technical
923 replicates ($n=4$). Data presented are representative of 3 independent experiments. **B)** Weight
924 loss and survival curve of WT C57BL/6J or IL1 receptor (*il1r*) knock-out ($^{-/-}$) mice after
925 intraperitoneal (IP) infection with 10^8 *L. interrogans* serovar Manilae strain L495 or control
926 PBS injection. **C)** Severity grading of clinical signs of WT C57BL/6J or *il1r* $^{-/-}$ mice after IP
927 infection with 10^8 *L. interrogans* serovar Manilae strain L495. **D)** Leptospiral loads measured
928 by qPCR in blood of surviving WT C57BL/6J or *il1r* $^{-/-}$ mice 3 days post IP infection
929 with 10^8 *L. interrogans* serovar Manilae strain L495 or control PBS injection. **E)** IL6 and IL1 β
930 cytokines mRNA levels measured by RT-qPCR in kidneys of surviving WT C57BL/6J or *il1r* $^{-/-}$
931 $^{-/-}$ mice 3 days post IP infection with 10^8 *L. interrogans* serovar Manilae strain L495 or control
932 PBS injection. **B-E)** Data were pooled from two independent experiments performed with
933 either $n=3$ male or $n=3$ female mice *per* group.

Figure 1.

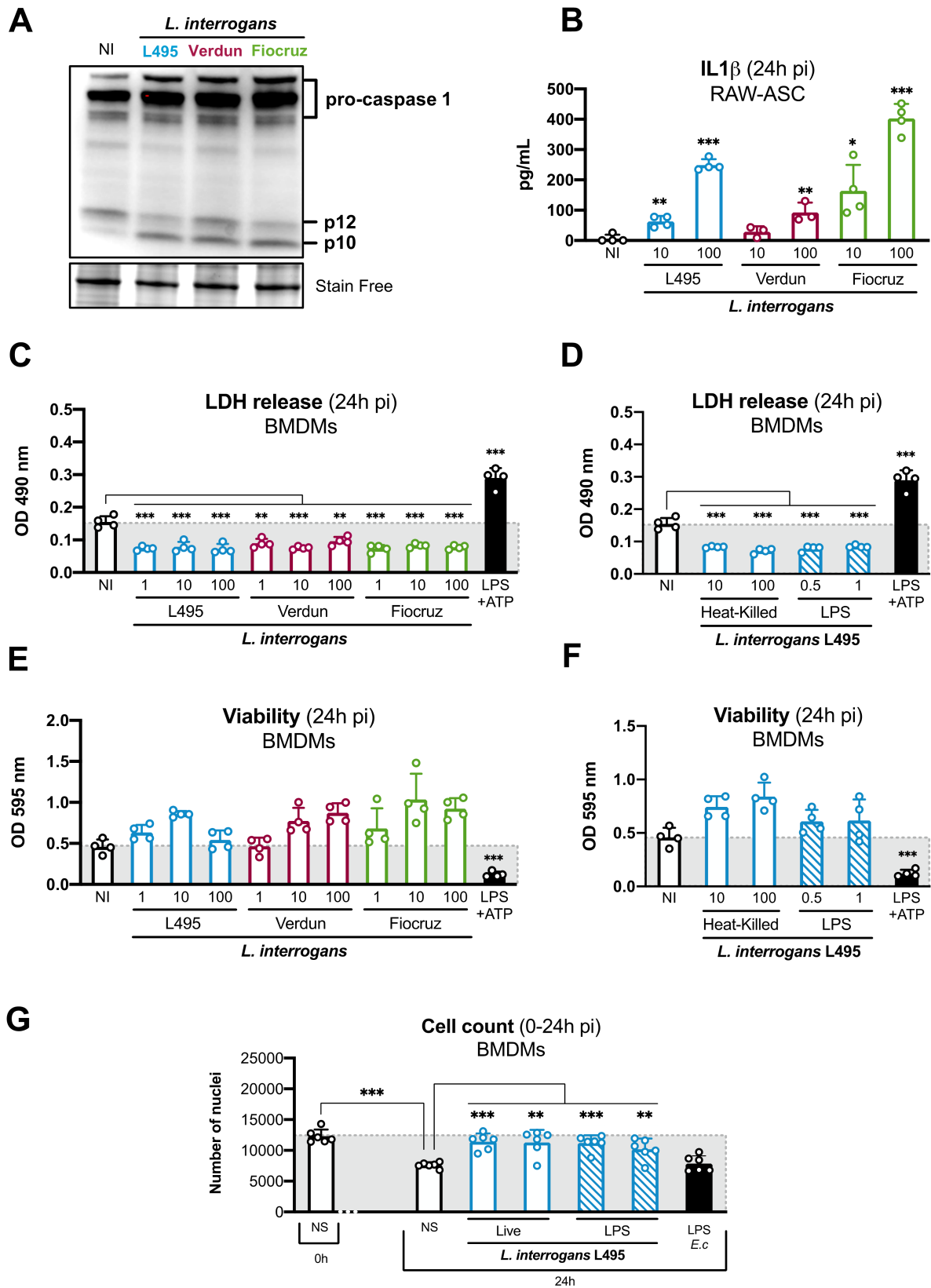


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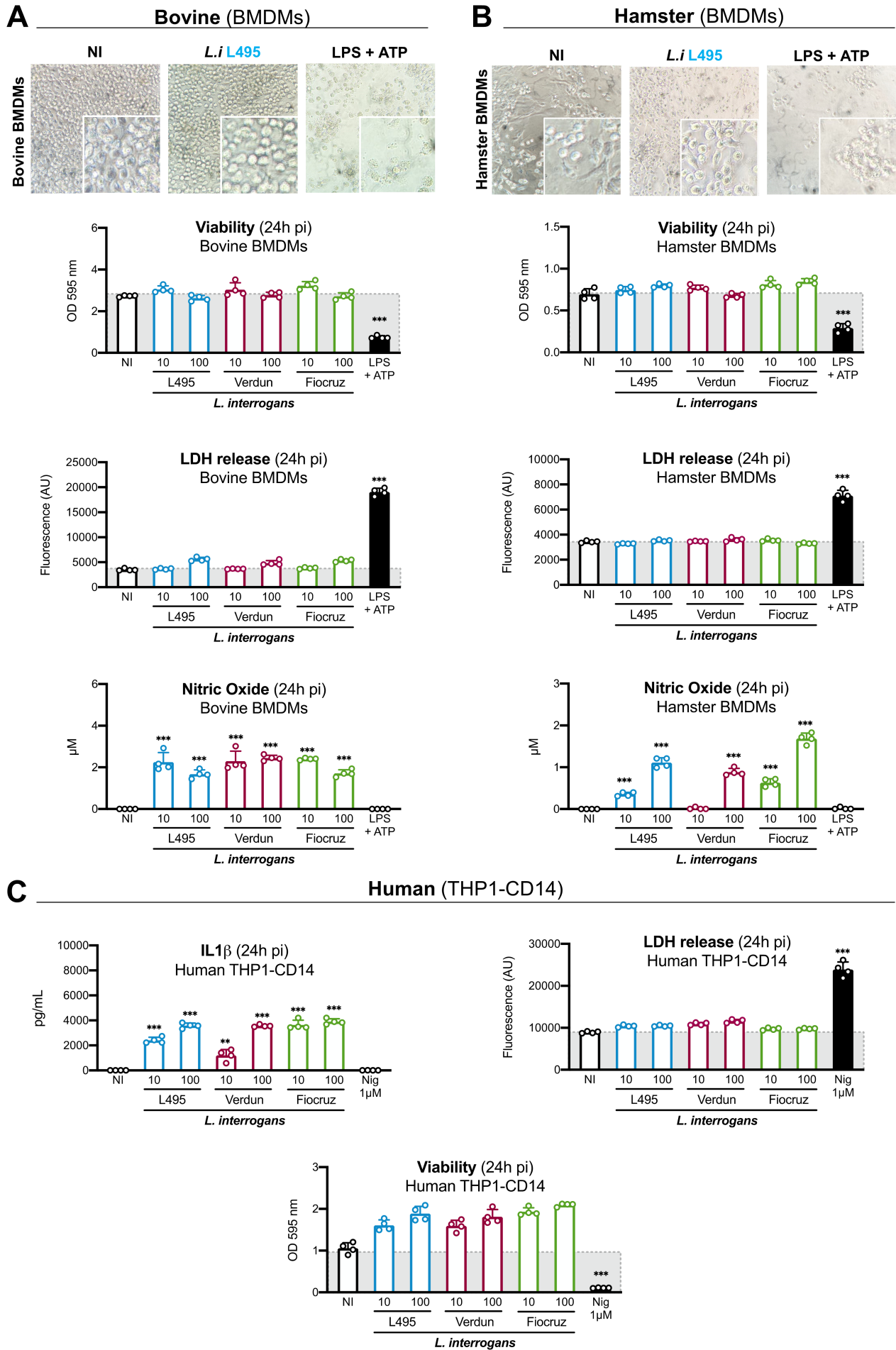


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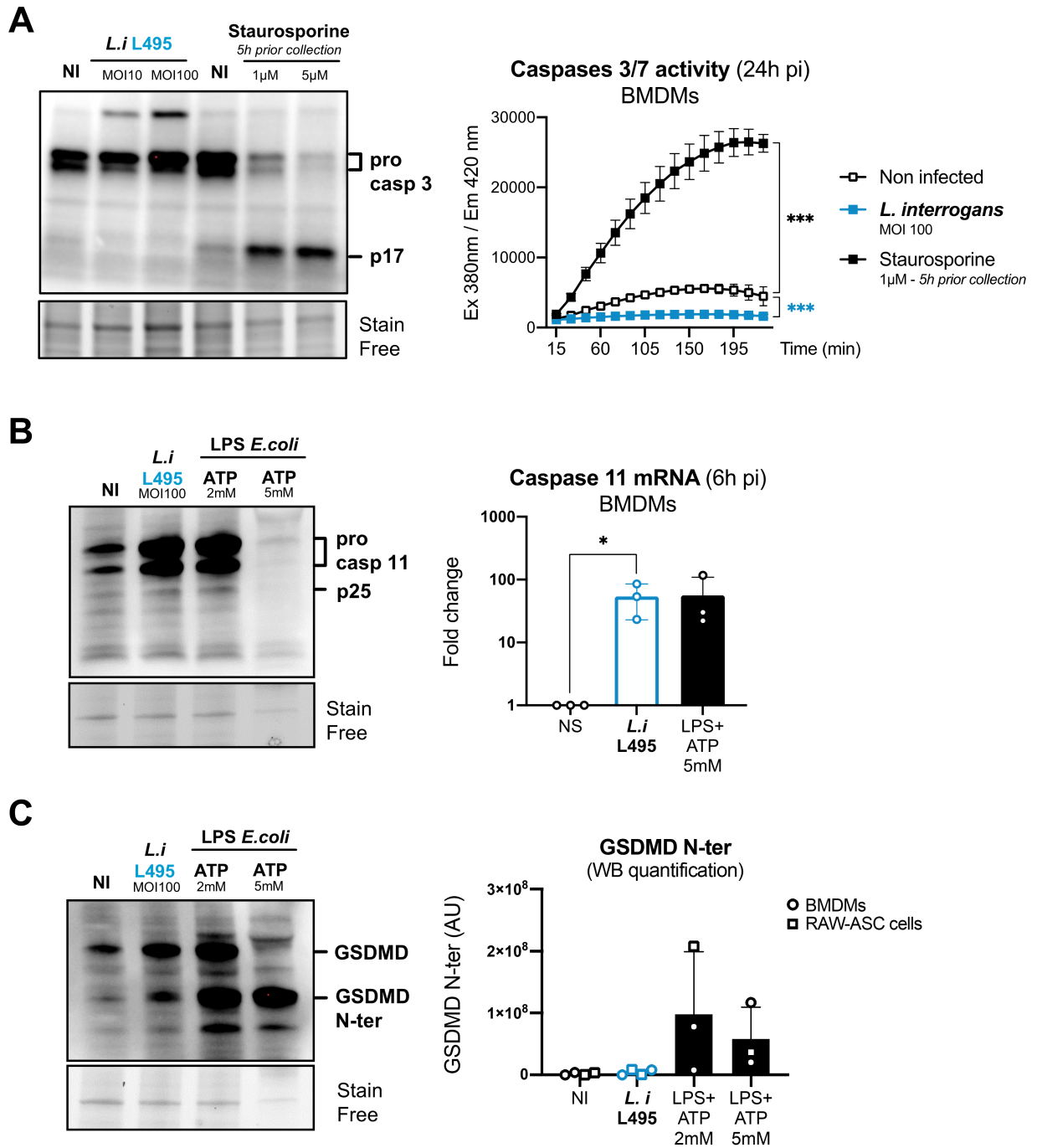
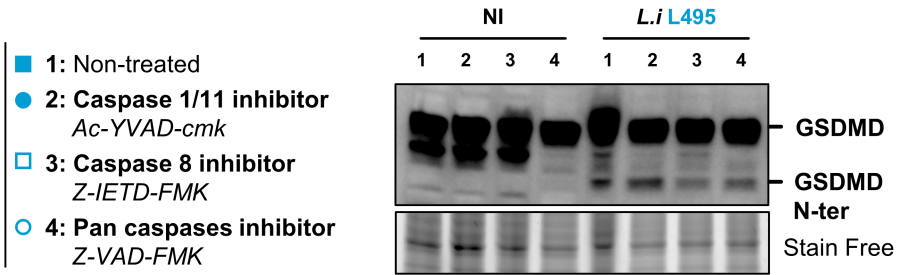
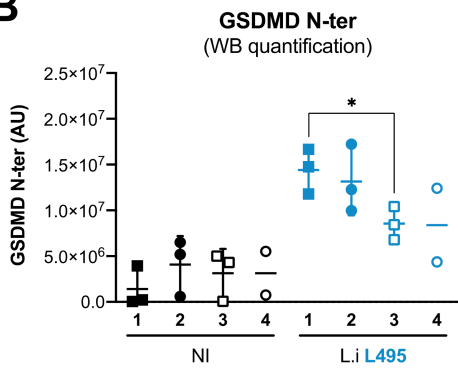


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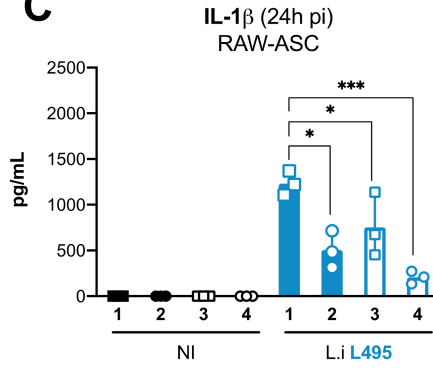
A



B



C



D

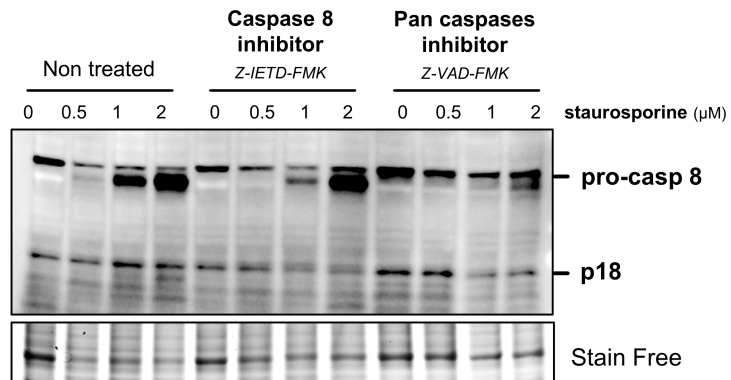
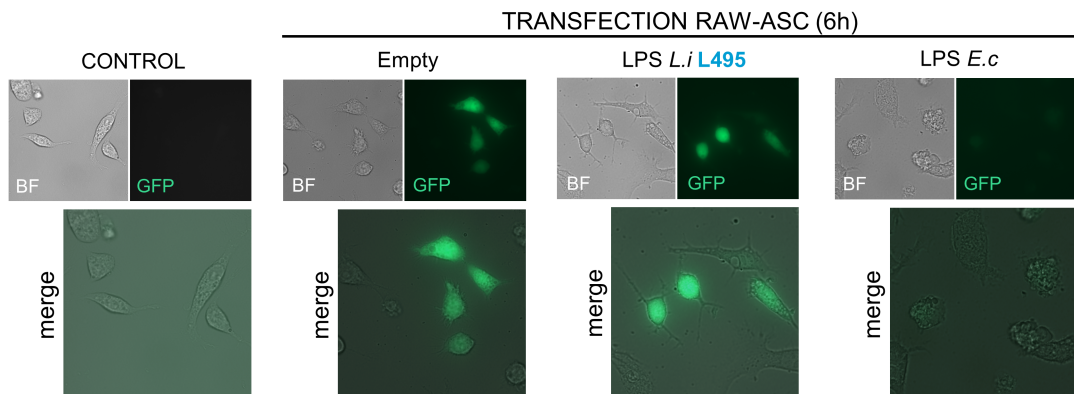
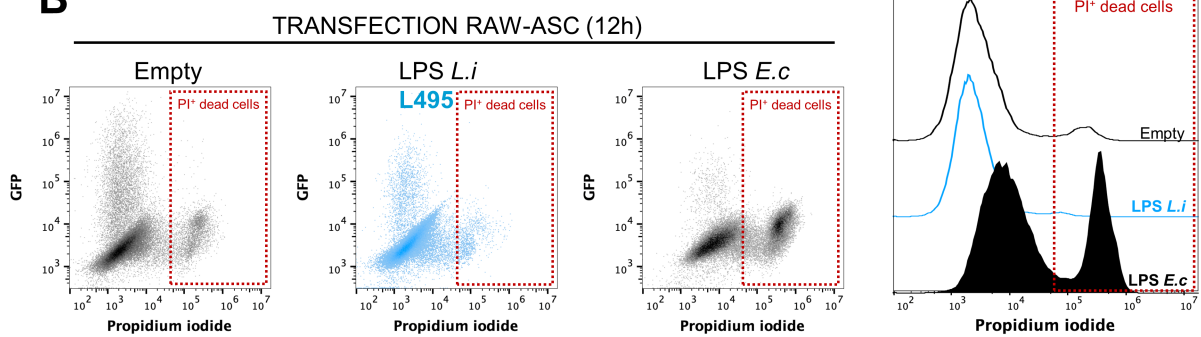


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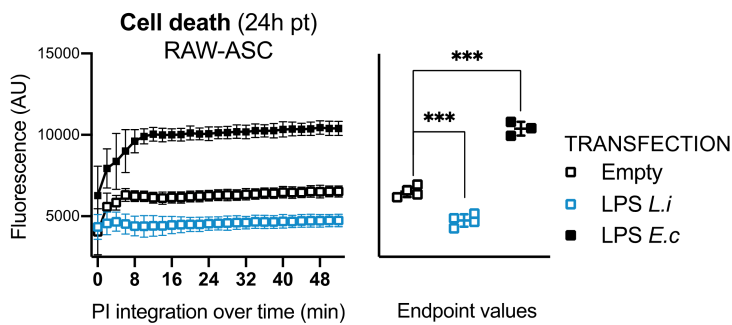
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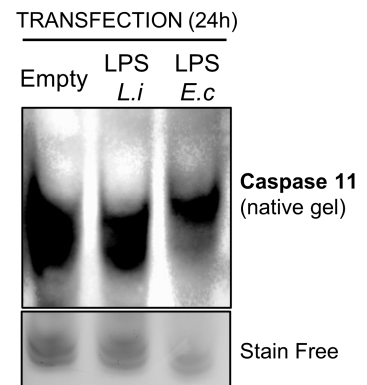
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C



E



D

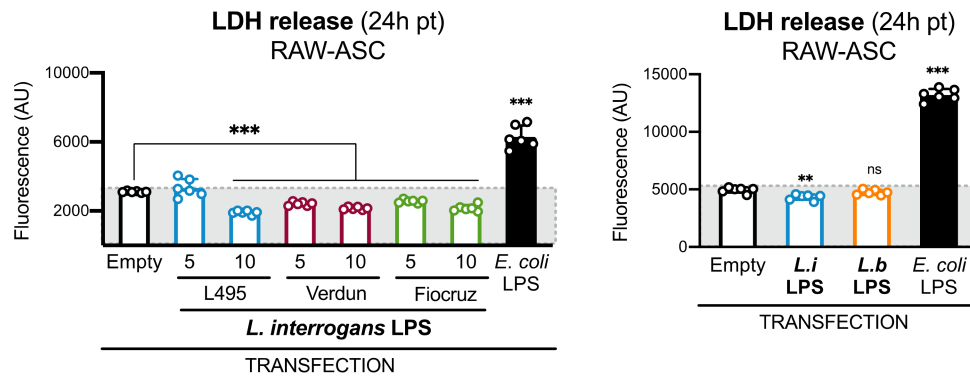


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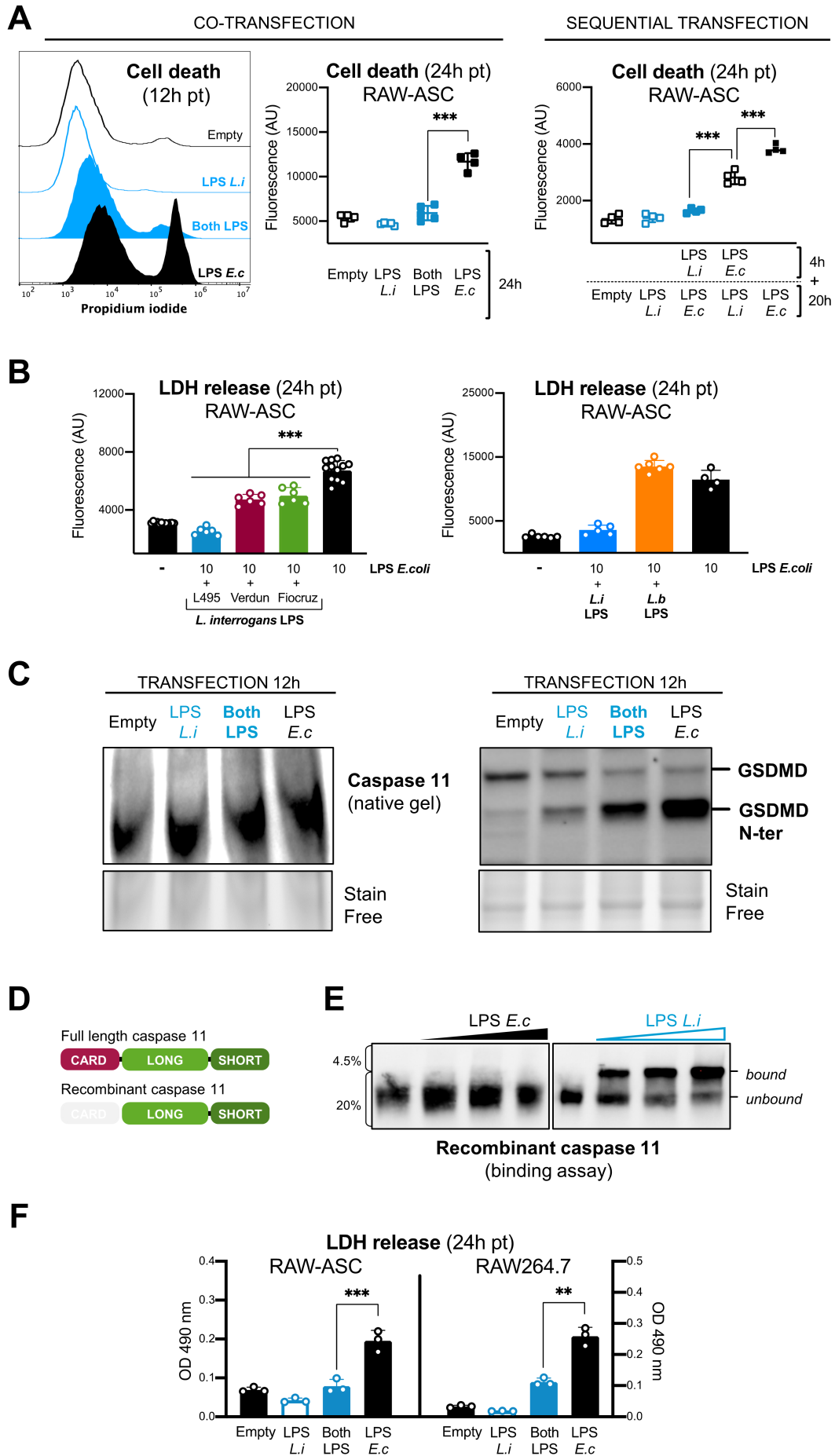
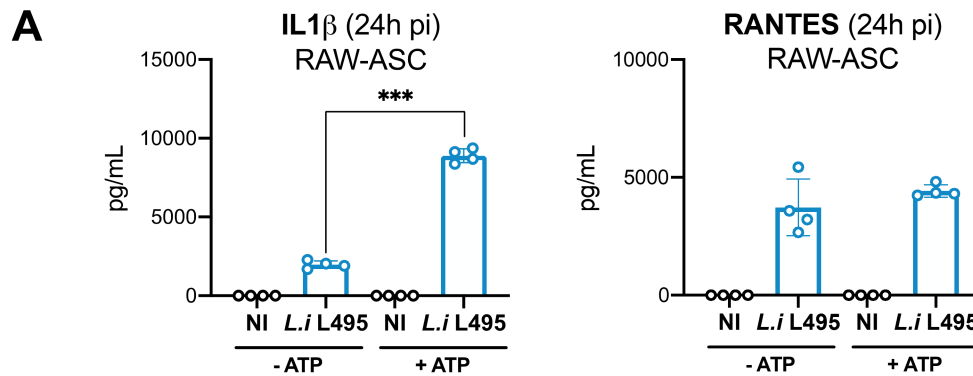
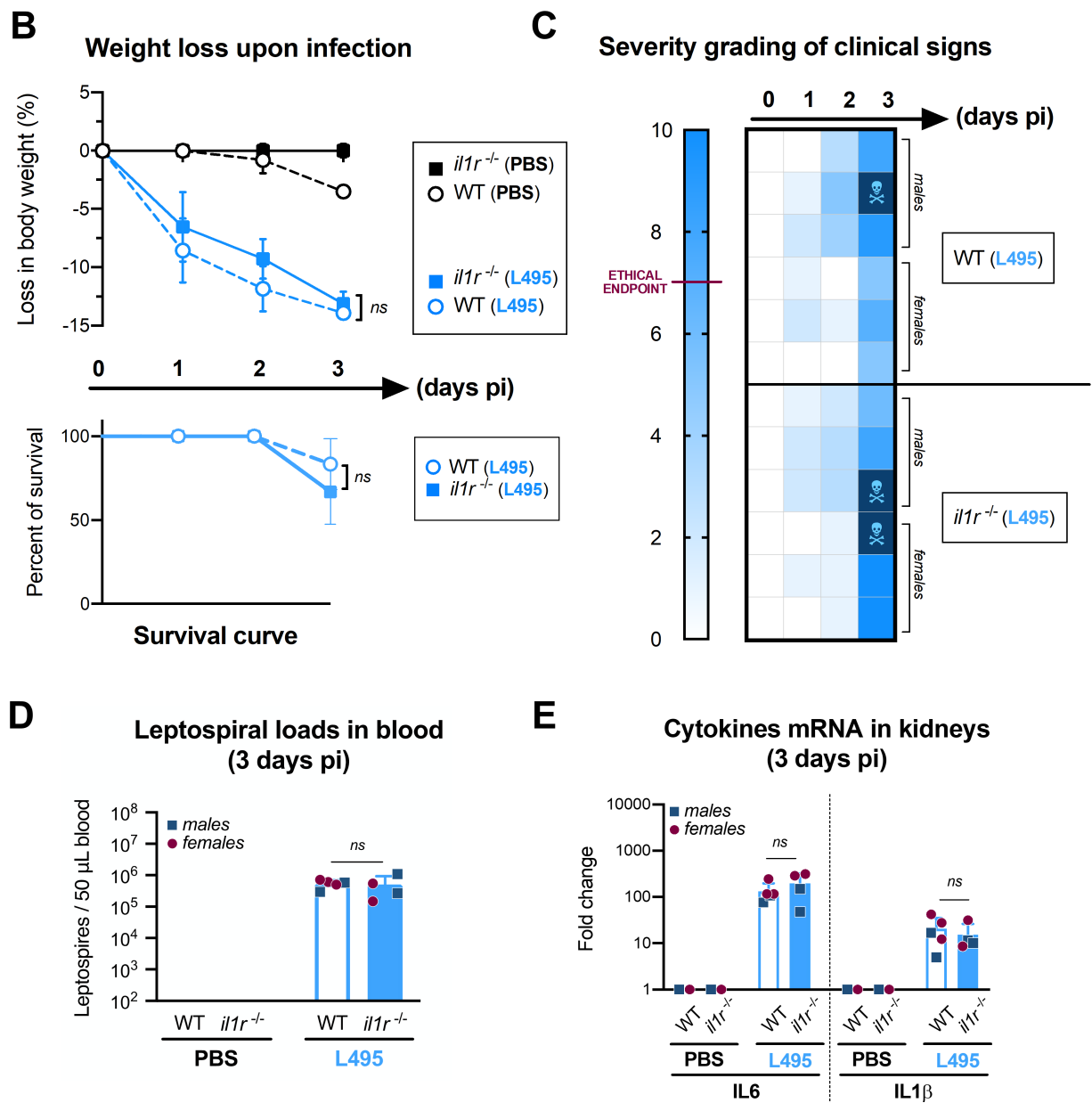


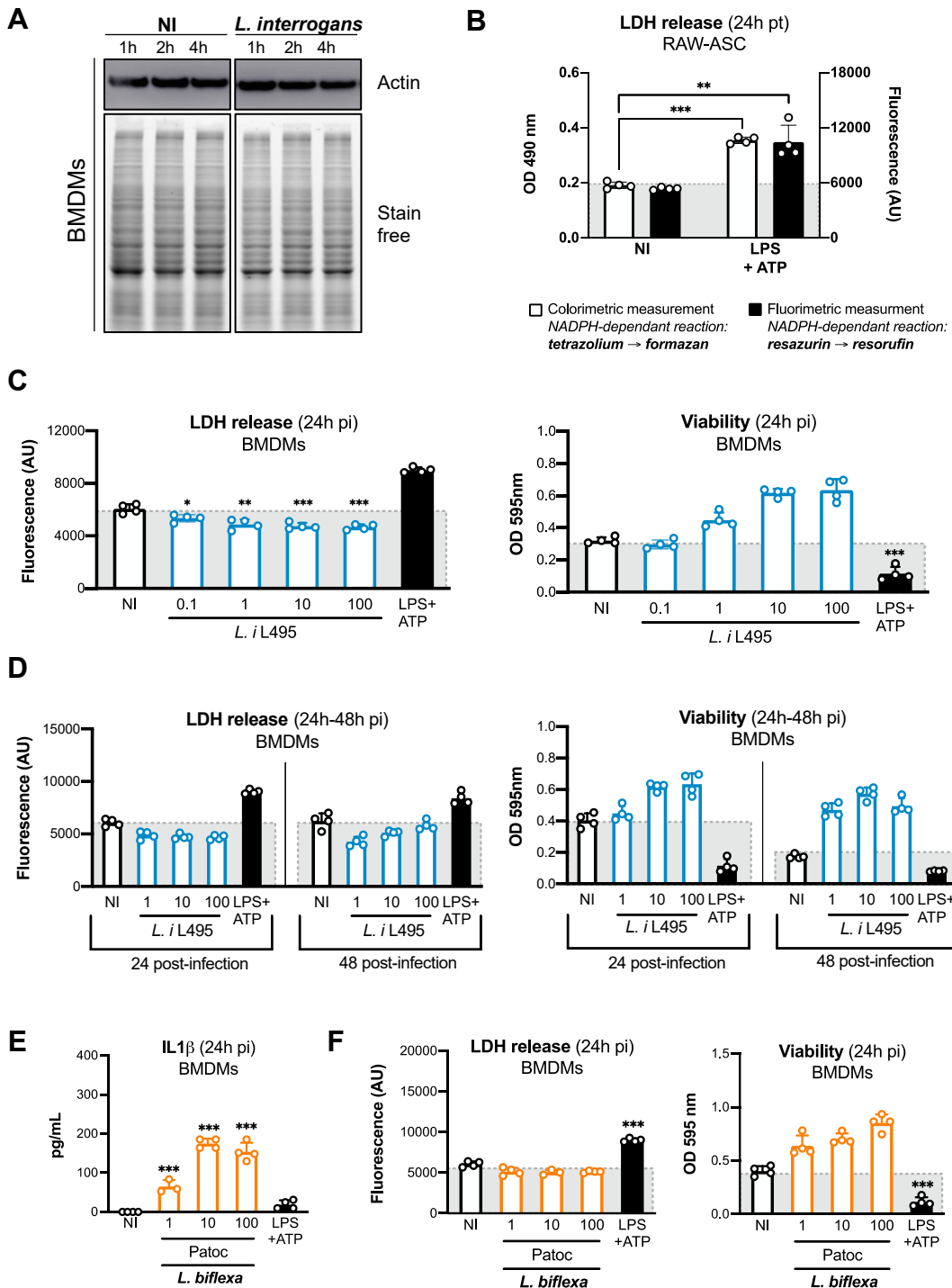
Figure 7.

In vitro, IL1 β release in murine macrophages



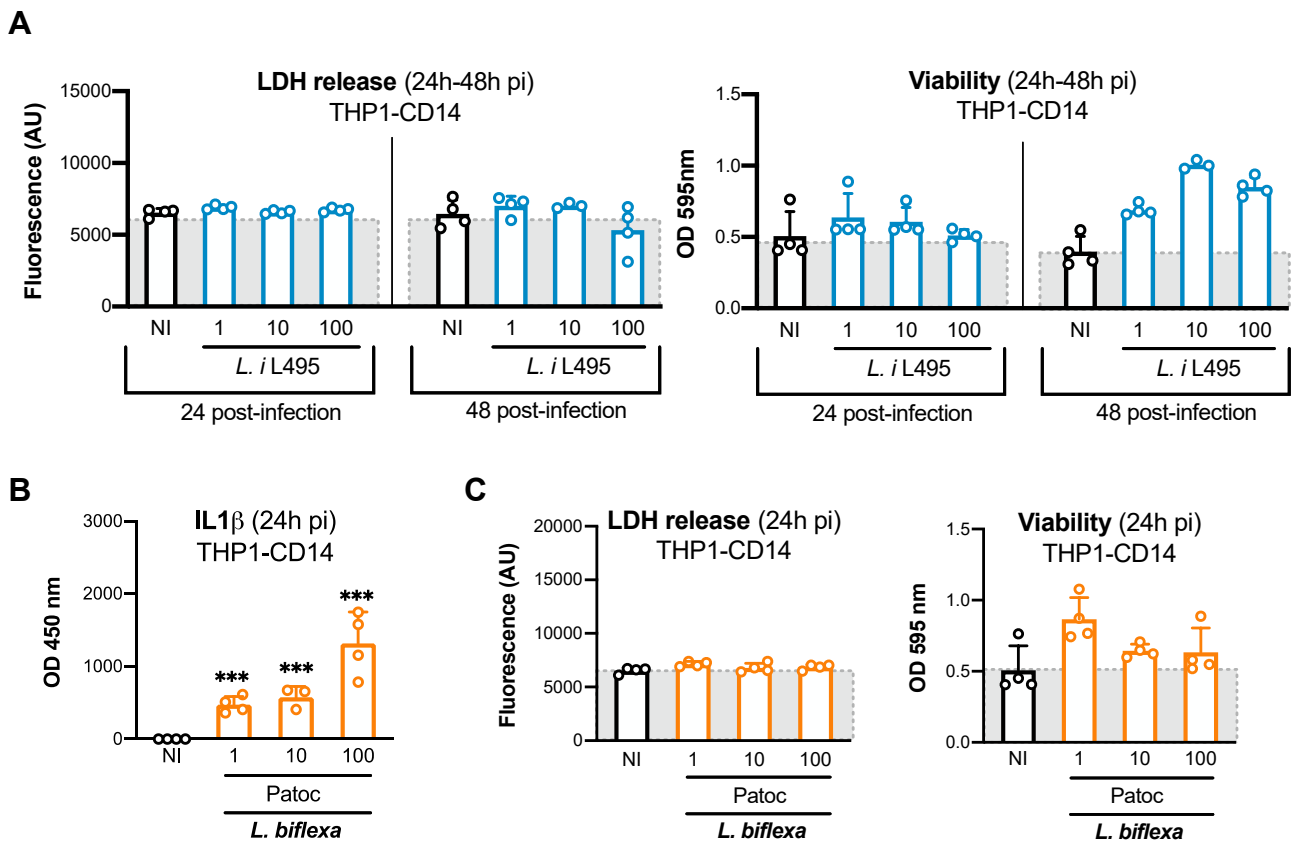
In vivo, experimental acute & lethal leptospirosis (10^8 leptospires L495 / mouse)





Supplementary Figure 1.

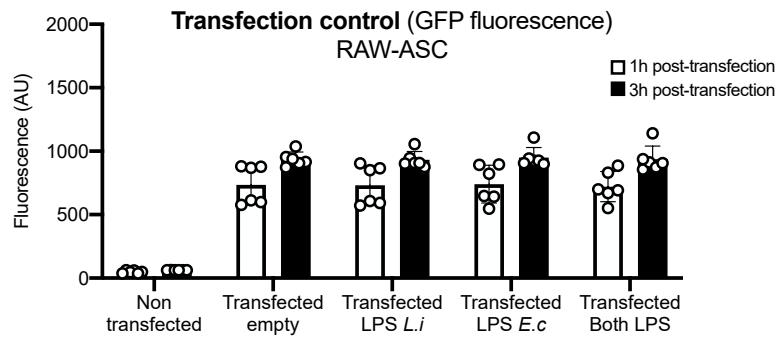
A) Western blot analysis of actin in BMDMs 1h, 2h and 4h post-infection with *L. interrogans* serovar Manilae strain L495. **B)** LDH release measured by both colorimetric and fluorimetric CyQuant assays on the supernatant of RAW-ASC cells 24h post-stimulation with 1 $\mu\text{g}/\text{mL}$ of *E. coli* LPS + 5 mM ATP. **C) Left panel.** LDH release (measured by CyQuant assay) on the supernatant of BMDMs after 24h infection with MOI 0.1-100 of *L. interrogans* serovar Manilae strain L495. **Right panel.** Cell viability (measured by MTT assay) in BMDMs after 24h infection with MOI 0.1-100 of *L. interrogans* serovar Manilae strain L495. Positive control of pyroptosis is 1 $\mu\text{g}/\text{mL}$ of *E. coli* LPS + 5 mM ATP. **D) Left panel.** LDH release (measured by CyQuant assay) on the supernatant of BMDMs after either 24h or 48h infection with MOI 1-100 of *L. interrogans* serovar Manilae strain L495. **Right panel.** Cell viability (measured by MTT assay) in BMDMs after either 24h or 48h infection with MOI 1-100 of *L. interrogans* serovar Manilae strain L495. Positive control of pyroptosis is 1 $\mu\text{g}/\text{mL}$ of *E. coli* LPS + 5 mM ATP. **E)** IL1 β dosage (measured by ELISA) in the supernatant of BMDMs after 24h infection with MOI 1-100 of *L. biflexa* strain Patoc I. **F) Left panel.** LDH release (measured by CyQuant assay) on the supernatant of BMDMs after 24h infection with MOI 1-100 of *L. biflexa* strain Patoc I. **Right panel.** Cell viability (measured by MTT assay) in BMDMs after 24h infection with MOI 1-100 of *L. biflexa* strain Patoc I.



Supplementary Figure 2.

A) Left panel. LDH release (measured by CyQuant assay) on the supernatant of THP1-CD14 cells after either 24h or 48h infection with MOI 1-100 of *L. interrogans* serovar Manilae strain L495. **Right panel.** Cell viability (measured by MTT assay) in THP1-CD14 cells after either 24h or 48h infection with MOI 1-100 of *L. interrogans* serovar Manilae strain L495.

B) IL1 β dosage (measured by ELISA) in the supernatant of THP1-CD14 cells after 24h infection with MOI 1-100 of *L. biflexa* serovar Patoc strain Patoc I. **C) Left panel.** LDH release (measured by CyQuant assay) on the supernatant of THP1-CD14 cells after 24h infection with MOI 1-100 of *L. biflexa* serovar Patoc strain Patoc I. **Right panel.** Cell viability (measured by MTT assay) in THP1-CD14 after 24h infection with MOI 1-100 of *L. biflexa* serovar Patoc strain Patoc I.

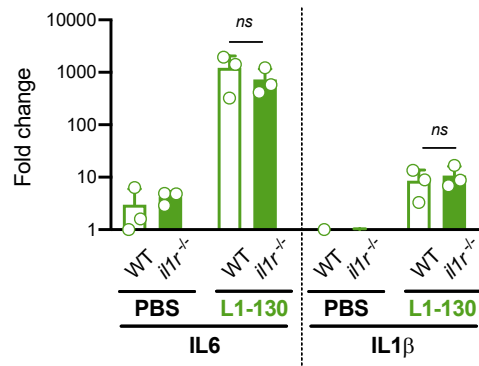


Supplementary Figure 3.

GFP-fluorescence analysis of RAW-ASC cells after 1h or 3h transfection with pCMV-GFP and 10 $\mu\text{g}/\text{mL}$ *E. coli* LPS, 10 $\mu\text{g}/\text{mL}$ *L. interrogans* LPS from serovar Manilae strain L495, or after co-transfection of 10 $\mu\text{g}/\text{mL}$ of both LPS.

In vivo, experimental acute leptospirosis
(2×10^8 leptospire Fiocruz L1-130 / mouse)

**Cytokines mRNA in kidneys
(3 days pi)**



Supplementary Figure 4.

IL6 and IL1β cytokines mRNA levels measured by RT-qPCR in kidneys in WT C57BL/6J or *illr*^{-/-} mice 3 days post IP infection with 2×10^8 *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 or control PBS injection. Data from one experiment performed with $n=3$ mice.