High level of IL-10 expression in the blood of animal models possibly relates to resistance against leptospirosis

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1 **Research article**

2

3 **Title: High level of IL-10 expression in the blood of animal models possibly relates to**
4 **resistance against leptospirosis**

5

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20

21 **Abstract**

22 Leptospirosis is a severe zoonosis which immunopathogenesis is poorly understood. We
23 evaluated correlation between acute form of the disease and the ratio of the anti-inflammatory
24 cytokine IL-10 to the pro-inflammatory TNF-α and IL-1β expression during the early phase of
25 infection comparing resistant mice and susceptible hamsters infected with two different species
26 of virulent *Leptospira*. The IL-10/TNF-α and IL-10/IL-1β expression ratios were higher in
27 mouse compared to hamster independently of the *Leptospira* strain, suggesting a preponderant
28 role of the host response and notably these cytokines in the clinical expression and survival to
29 leptospirosis. Using an IL-10 neutralization strategy in *Leptospira*-infected mouse model, we
30 also showed evidence of a possible role of this cytokine on host susceptibility, bacterial
31 clearance and on regulation of cytokine gene expression.

32

33 **Keywords:** leptospirosis; *in vivo;* cytokine; IL-10; IL-1β; TNF-α

34

35 **Highlights**

36 • Higher IL-10/TNF-α and IL-10/IL-1β expression ratios in mouse compared to hamster.
37 • Differential expression independently of the *Leptospira* strain.
38 • Neutralisation of IL-10 led to increased susceptibility in *Leptospira*-infected mouse.
39 • IL-10 has a role in regulation of IL-1β during leptospirosis.
40 • IL-10 neutralization did not interact with TNF-α expression level.

41

42

43 ## 1. Introduction

44 Mainly reported in tropical and developing countries, leptospirosis is a zoonosis caused by
45 pathogenic *Leptospira*, and recent estimations revised the annual number of human cases over
46 one million infection cases with almost 60,000 deaths [1]. Contamination occurs after contact
47 with bacteria shed in the urine of reservoir animals, mostly rodents [2] and human leptospirosis
48 is characterized by various clinical symptoms that can degenerate to severe forms with
49 haemorrhages and multiple organ failure [3].

50 Severe human leptospirosis presents evidence of a "cytokine storm" process
51 characterized by increased levels of pro- (TNF-α, IL-6) and anti- (IL-10) inflammatory
52 cytokines in serum of patients [3] and several inflammatory mediators were investigated as
53 biomarkers of severity in human cases. Interestingly, high ratio of IL-10 to TNF-α circulating
54 level was correlated to severity of human leptospirosis [4, 5]. However, contradictory results
55 were also reported showing that higher ratio of IL-10 to TNF-α serum levels was positively
56 correlated with fatal outcome [6, 7]. Interestingly, the last study of Chirathaworn and coll. did
57 not show significant difference of IL-10/TNF-a ratio in leptospirosis patients with or without
58 organ involvement [8]. Previously, we demonstrated that gene expression levels of TNF-α and
59 IL-10 in blood were significantly higher and related to lethality in a susceptible infection model
60 using hamsters [9]. We also studied the cytokine gene expression in the blood of resistant mice,
61 and, while pro-inflammatory cytokines were restored to basal levels during the early days
62 postinfection, IL-10 was rapidly overexpressed [10].

63 In this study, we performed further experiments *in vivo* to clarify the regulation of
64 cytokines IL-10, IL-1β and TNF-α, in animal models of experimental leptospirosis, also
65 investigating tissue lesions and bacterial burden. Mice were used as asymptomatic models,
66 either regarded as resistant when infected with *L. interrogans* serovar Icterohaemorrhagiae
67 strain Verdun (LiVV) or as a typical reservoir model when inoculated with a *L. borgpetersenii*

68 serogroup Ballum. Hamsters were used to reproduce severe leptospirosis when infected with
69 either *Leptospira* strain. As infecting serovars can affect the severity of human leptospirosis
70 [11], two different species of virulent leptospires were used for infection. Moreover, to better
71 assess the acute phase of infection in the susceptible model, gene expression was investigated
72 between 3 and 4 days (D) postinfection in the blood of animals, also corresponding to high
73 leptospiremia in susceptible hamsters. The ratios of IL-10/TNF-α and IL-10/IL-1β were
74 investigated according to animal survival or fatal outcome, histological changes in organs and
75 bacterial burden in blood. We then investigated the possible involvement of the
76 immunosuppressive IL-10 in leptospirosis pathogenesis using an IL-10 neutralization strategy
77 in *Leptospira*-infected mouse model to monitor its possible effect on host susceptibility,
78 bacterial clearance and on regulation of cytokine gene expression.

79

80 ## 2. Material and Methods

81 ### 2.1. Animals and bacterial strains

82 Outbred OF1 mice (*Mus musculus*) and golden Syrian hamsters (*Mesocricetus auratus*),
83 initially obtained from Charles River Laboratories, were bred in animal facility in Institut
84 Pasteur in New Caledonia. Virulent *L. interrogans* serovar Icterohaemorrhagiae strain Verdun
85 (LiVV) or *L. borgpetersenii* serogroup Ballum isolate B3-13S (B3-13S) were cultured in EMJH
86 medium as previously described [12]. Virulence of leptospires was maintained and verified by
87 monthly passages in 6- to 8-week-old golden Syrian hamsters after intraperitoneal injection
88 before re-isolation from blood. Animal manipulations were conducted according to the
89 guidelines of the Animal Care and Use Committees of the Institut Pasteur and followed
90 European Recommendation 2007/526/EC.

91

92 ### 2.2. Comparative experimental infection

93 For comparative study between *Leptospira* strains, 6- to 8-week-old healthy animals ($n \geq 5$
94 individuals per condition) were infected and experiments were carried out as previously
95 described [12]. Briefly, mice and hamsters were injected intraperitoneally with 2×10^8
96 leptospires in EMJH medium. Non-infected animals served as controls. After euthanasia with
97 chloroform or by inhalation of CO_2, whole blood was collected between D3 and D4
98 postinfection by cardiac puncture and conserved in PAXgene blood RNA tubes (PreAnalytiX,
99 Qiagen) for molecular biology. Sera of LiVV-infected mice were collected at D3 and D7 after
100 infection for protein quantitation. Organs were fixed in 10% neutral buffered formalin for
101 histology.

102

103 **2.3. IL-10 neutralization challenge**

104 For evaluation of IL-10 neutralization in murine models, 5-week old OF1 mice were injected
105 with 200 µg of anti-IL-10 antibodies [13] in 200 µL of PBS (LEAF Purified anti-mouse IL-10,
106 rat IgG1, κ from clone JES5-2A5, or rat IgG2b, κ from clone JES5-16E3, BioLegend, Australia)
107 3 hours prior to infection with 2×10^8 leptospires of the strain LiVV in EMJH medium (αIL-
108 10+LiVV group). Infected animals without antibodies treatment were injected with 200 µL of
109 PBS (PBS+LiVV group) to reproduce double injection. Mice injected with anti-IL-10 and
110 EMJH medium were also included (αIL-10+EMJH group). Animals without injection served
111 as control. One hamster was also infected with the same dose of bacteria to confirm virulence
112 and lethality of the strain during experiment. Body weight of mice was monitored prior to
113 injections and until D7 postinfection. Animals were observed for survival and for any signs of
114 clinical illness (loss of appetite or reactivity, prostration, ruffled fur, external hemorrhage).
115 Blood samples (50 µL) were non-lethally collected by caudal vein puncture at D3 postinfection
116 and processed as detailed above using the PAXgene blood RNA system (PreAnalytiX, Qiagen).
117 Animals were humanely killed at D7 postinfection and kidneys were rapidly collected for

118 molecular bioassays (within 5 minutes of euthanasia) for extraction of total DNA. Briefly, 25
119 mg samples were placed into MagNA Lyser Green Beads tubes (Roche Applied Sciences)
120 containing 360 µL lysis buffer (QIAamp DNA Mini kit, Qiagen).
121
122 **2.4. Histology**
123 Paraffin-embedded sections were stained with hematoxylin-erythrosin (HE). Morphological
124 changes in liver, lungs and kidneys were analyzed and lesions were graded blindly according
125 to previously reported criteria [10]. Average scores were totalized ranging from normal (0) to
126 severe (20) for each individual.
127
128 **2.5. Purification of DNA**
129 Total DNA was extracted using QIAamp DNA Mini kit (Qiagen) from renal tissues. Tissue
130 samples in MagNA Lyser Green Beads tubes were first disrupted and homogenized using the
131 MagNA Lyser Instrument (Roche Applied Science) during 50 s at 7,000 r/min. Lysates were
132 incubated at 56°C for 1 h with 190 µL of PBS and 50 µL of proteinase K. After washing steps,
133 the eluted DNA was quantified by spectrophotometry (NanoDrop 2000, ThermoFisher).
134
135 **2.6. Reverse Transcription**
136 For gene expression, total RNA was purified using the PAXgene blood RNA system before
137 treatment with DNase (Turbo DNA-free kit; Ambion, Applied Biosystems), and cDNA
138 synthesized using Transcriptor First Strand cDNA synthesis kit (Roche Applied Science) as
139 previously described [10].
140
141 **2.7. Quantitative PCR**

142 Primers and probes were purchased from Eurogentec (Seraing, Table 1). Renal DNA was
143 amplified using the LightCycler FastStart DNA Master SYBR Green I and targeted the *lfb1*
144 gene [14] on a LightCycler 2.0 (Roche Applied Science). Alternatively, bacterial load was
145 assessed using the LightCycler 480 Probe Master targeting *lipL32* gene on a LightCycler 480
146 II instrument (software v.1.5.0; Roche Applied Science) [15]. Genomic DNA from
147 corresponding leptospires was used as a positive control. Quantitative PCR for IL-10, IL-1β,
148 TNF-α, *Leptospira* 16S rRNA and the reference genes glyceraldehyde-3-phospho-
149 deshydrogenase (GAPDH) and β–actin were conducted on the LightCycler 480 II using the
150 cDNA previously generated and the LightCycler 480 SYBR Green I Master kit as previously
151 described [10]. Normalization of expression was processed using qbase PLUS software
152 (Biogazelle) by extracting the expression levels of the reference genes. The normalized
153 expression ratio of *Leptospira* 16S rRNA was calculated for evaluation of bacteria level. For
154 cytokine gene expression, the relative normalized expression ratio was then calculated as the
155 ratio of the expression level in infected animals to the expression level in control animals.
156
157 **2.8. Quantification of cytokines (xMAP technology)**
158 Interleukin-10, IL-1β and TNF-α levels in serum of mouse were quantified using a multiplex
159 magnetic bead-based immunoassay (MyCytoMag 70k-10.Mouse kit, Merck-Millipore),
160 according to manufacturer's instructions. The specific bead fluorescence was measured by a
161 MAGPIX® instrument (Luminex, Austin, USA). The sample concentrations were calculated
162 using a 10-parameter logistic standard curve using the MILLIPLEX® Analyst 5.1 software
163 (Merck Millipore, Billerica, USA).
164
165 **2.9. Statistical analyses**

166 Statistical studies were performed using GraphPad Prism v4 (GraphPad Software Inc.). Mann-
167 Whitney test was used for analysis of significant differences in histopathological score,
168 bacterial load and cytokine gene expression between animals. A Two-way ANOVA test was
169 used to compare difference in body weight variation depending on treatment between mice
170 during IL-10 neutralization challenge.

171

172 # 3. Results

173 ## 3.1. Tissue lesions and leptospiremia in *Leptospira*-infected animals

174 Animals were infected with LiVV or B3-13S strain and fatal outcome was observed in hamsters
175 within four to six days after inoculation as previously shown [9, 12]. Severe alterations were
176 observed in B3-13S-infected hamster organs with diffuse alveolar haemorrhages, oedema and
177 pulmonary congestion with inflammatory infiltrates in lungs (Figure 1A), interstitial nephritis,
178 glomerular congestion and tubular haemorrhages in kidneys (Figure 1B), and hepatocytic
179 necrosis, sinusoidal congestion (Figure 1C), portal inflammation and hyperplasia of Kupffer
180 cells in liver. Similar observations were reported in LiVV-infected hamsters (data not shown).
181 In contrast, no or mild morphological changes were observed in *Leptospira*-infected mice (data
182 not shown).

183 Histopathological scores (Figure 1D) showed significantly more severe lesions in hamsters
184 compared to mice infected with LiVV (mean score ± SE at 5.33 ± 0.95 and 13.17 ± 1.33 in mice
185 and in hamsters, respectively) or with B3-13S (0.15 ± 0.15 and 7.0 ± 3.41 in mice and in
186 hamsters, respectively). Bacterial burden was investigated in the blood of animals during the
187 onset of the symptoms between three and four days postinfection (Figure 1E) and, as expected,
188 bacterial load was significantly higher in hamster blood (mean ± SE at 17.06 ± 6.31 and
189 0.57 ± 0.18 for infection with LiVV and B3-13S, respectively) compared to mice (3.41 ± 1.20
190 and <1E-4 for infection with LiVV and B3-13S, respectively) regardless of the infecting strain.

191

192 **3.2. Cytokine gene expression in blood of animals depending on infecting strains**

193 Gene expression level of cytokines was quantified in the blood of animals and IL-10 gene
194 expression (Figure 2A) was significantly higher at D3-D4 postinfection in the blood of infected
195 mice compared to hamsters (mean ratios of 18.95 ± 7.15 compared to 5.07 ± 1.51 respectively
196 for LiVV infection or 80.19 ± 21.67 compared to 17.34 ± 3.33 respectively for B3-13S
197 infection). Significant overproduction of IL-10 was confirmed in sera of mice infected with
198 LiVV 3 days after infection (265.0 ± 89.72 pg/mL) compared to control level (7.23 ± 0.48
199 pg/mL; Figure 2D). Blood IL-10 returned to basal level at D7 postinfection (8.12 ± 1.38
200 pg/mL). Unfortunately, commercial kits are not available for quantification of cytokines in the
201 blood from Syrian hamster.

202 Gene expression of TNF-α was also significantly up-regulated at D3-D4 in both mice and
203 hamsters (Figure 3A) infected with LiVV (means ± SE of expression ratio at 2.46 ± 0.84 and
204 3.31 ± 0.40 in mice and hamsters, respectively) or with B3-13S strain (9.26 ± 2.59 and
205 15.07 ± 2.70 in mice and hamsters, respectively). Interestingly, no difference was observed
206 between animals irrespective of strains. All IL-1β expression levels were up-regulated and
207 significantly different compared to control in animals infected with LiVV (ratio at 3.75 ± 1.8 in
208 hamsters) or with B3-13S strain (10.06 ± 4.66 in mice and 9.95 ± 5.17 in hamsters,
209 respectively), except for LiVV-infected mice (Figure 3B). However, contrasting with TNF-α,
210 significant difference was observed in IL-1β expression between mice and hamsters during
211 LiVV infection, with higher expression in hamsters.

212 Both IL-10/TNF-α and IL-10/IL-1β ratios of gene expression values in blood (Figure 3C-
213 D) were significantly higher in mice (25.13 ± 11.90 and 11.91 ± 3.44 with LiVV and B3-13S,
214 respectively for IL-10/TNF-α, and 13.99 ± 7.49 and 7.99 ± 4.21 with LiVV and B3-13S,
215 respectively for IL-10/IL-1β) than in hamsters (1.44 ± 0.29 and 2.39 ± 0.99 with LiVV and B3-

216 13S, respectively for IL-10/TNF-α ratio, and 1.31 ± 0.51 and 2.626 ± 1.19 with LiVV and B3-
217 13S, respectively for IL-10/IL-1β). Interestingly, cytokine gene expression level was
218 significantly different depending on the strain used for infection among mice or hamsters
219 (Figure 3A-B) but no effect of strain was observed when comparing expression ratios IL-
220 10/TNF-α or IL-10/IL-1β (Figure 3C-D). Cytokine concentrations in sera of mice were also
221 quantified using multiplex technology, and TNF-α and IL-1β were not different between control
222 and infected mice at D3 postinfection possibly related to low expression level (data not shown).
223

224 **3.3. Characterization of LiVV infection after IL-10 neutralization in mice**

225 In order to investigate the role of IL-10 in pathogenesis, anti-IL-10 neutralizing antibodies were
226 injected mice prior to their infection. LiVV strain was used and animals were double injected
227 either with PBS or with EMJH to evaluate potential activity of solutions. All mice survived
228 during experimental infection. However, lower reactivity and mobility, and loss of appetite
229 were observed in LiVV-infected mice challenging IL-10 neutralization during the first 2 days
230 postinfection while no clinical signs were observed for the other animal groups. Body weight
231 of animals (Figure 4A) was monitored to assess the effect of IL-10 neutralization on
232 leptospirosis severity and bacterial load. No significant difference was observed for double
233 injected PBS+LiVV or for αIL-10+EMJH mice compared to non-injected control animals,
234 confirming natural resistance of OF1 mouse to LiVV infection. In contrast, αIL-10+LiVV mice
235 showed a significant decrease in body weight (percentage of initial body weight of 94.14 ±
236 2.80 % and 98.43 ± 3.17 % at D1 and D2 postinfection, respectively) when compared to
237 PBS+LiVV injected animals (99.42 ± 2.78 and 103.3 ± 3.60 at D1 and D2 postinfection,
238 respectively).

239 Leptospiremia was also measured in blood of infected mice and no difference was observed
240 during IL-10 neutralization challenge at D3 postinfection (Figure 4B). Presence of leptospires

241 was investigated and bacterial load was also quantified in kidneys of animals at D7
242 postinfection (Figure 4C). Interestingly, leptospires were detected in 1 / 7 mice in kidneys from
243 LiVV-infected mice pre-injected with anti-IL-10 antibodies while 5 / 7 animals were positive
244 in PBS+LiVV group. Moreover, leptospiral load was lower in αIL-10+LiVV mice (6.27 ±
245 16.59) compared to PBS+LiVV animals (552.20 ± 760.80) supporting a possible effect of IL-
246 10 neutralization on bacterial dissemination or clearance in mice kidneys.
247

248 3.4. Effect of IL-10 neutralization on *Leptospira*-dependent cytokine gene expression in 249 mouse

250 Regulation of cytokine gene expression was investigated in blood of LiVV-infected mice
251 challenging IL-10 neutralization at D3 postinfection (Figure 5). Up-regulation of IL-10 gene
252 expression was confirmed in infected animals compared to control group (mean expression
253 ratio at 14.98 ± 3.87 in PBS+LiVV group; Figure 5A) without significant effect of anti-IL-10
254 antibodies treatment on LiVV-dependent gene overexpression (ratio at 10.84 ± 4.72). Similarly,
255 TNF-α was overexpressed in blood of infected mice compared to control but no significant
256 difference was observed between LiVV-infected animals with or without anti-IL-10 antibodies
257 pre-injection (Figure 5B). A different expression pattern was observed for IL-1β transcript level
258 (Figure 5C) with no significant overexpression of this cytokine in blood from PBS+LiVV group
259 (mean expression ratio at 1.45 ± 0.32) compared to control animals, comforting previous results
260 (Figure 3B). In contrast, IL-1β gene expression was significantly upregulated in blood from
261 LiVV-infected mice challenging IL-10 neutralization (ratio at 2.12 ± 0.24) compared to control
262 group, and significantly different compared to PBS+LiVV animals.
263

264 4. Discussion

265 Cytokines constitute key inflammatory mediators that orchestrate immune responses to
266 infection [16] and differential regulation of cytokine expression was related to clinical outcomes
267 in human leptospirosis. Expression levels of cytokines including IL-10, IL-1β and TNF-α were
268 previously evaluated as potential markers of leptospirosis severity in human, and studies
269 showed contrasting results regarding ratio of IL-10 to TNF-α correlated with disease severity
270 [4-8]. This might be explained by difference in infecting strains. Indeed, using a hamster model,
271 Fujita and collaborators showed differential regulation of cytokine gene expression depending
272 on the infecting serovars [17]. Thus, we analysed regulation of IL-10, IL-1β and TNF-α in blood
273 from susceptible hamsters and resistant mice infected with two different species of leptospires
274 to investigate effect of strains on cytokine expression, and relation between animal resistance
275 and cytokine regulation. Except for IL-1β in LiVV-infected mice, gene expression of cytokines
276 was up-regulated in blood of animals at D3-4 postinfection compared to control level.
277 Interestingly, IL-10, IL-1β and TNF-α expression levels but not the IL-10/TNF-α nor IL-10/IL-
278 1β expression ratios were differentially regulated within the same animal model depending on
279 inoculated leptospires. Infecting strains might differentially affect each cytokine gene
280 expression while IL-10/TNF-α and IL-10/IL-1β ratios appear independent of leptospire
281 serovars, suggesting these ratios as possible indicators of leptospirosis severity.

282 Less tissue lesions and lower leptospiremia with higher cytokine expression levels were
283 observed in B3-13S- compared to LiVV-infected animals and it is noticeable that B3-13S
284 leptospires belong to the serovar Ballum which natural reservoirs are mice [11]. Thus, infection
285 of mice with their host-coadapted serovar also promotes inflammatory responses with
286 upregulation of cytokine levels during the early phase of infection. This also suggests virulence
287 of strain as a preponderant factor of cytokine regulation possibly affecting observations and
288 prognosis in human cases as previously shown [18]. Conversely, cytokine regulation could be
289 regarded as a major actor of leptospirosis pathophysiology. Pro-inflammatory TNF-α and anti-

290 inflammatory IL-10 are amongst the main biological mediators exerting adverse effects during
291 bacterial infection and both cytokines are induced during experimental leptospirosis as
292 previously shown [10]. However, no difference was observed in TNF-α expression level
293 between hamster and mouse when infected with the same strain. This correlates with
294 observations in leptospirosis patients of whom TNF-α although induced in blood, was not
295 different depending on organ involvement or severity [5, 8]. Oppositely, IL-10 level was higher
296 in mouse blood compared to hamster blood irrespective of the strain. This suggests a
297 preponderant role of this anti-inflammatory cytokine in the resistance of mice to leptospirosis.
298 That is reflected by a higher IL-10/TNF-α and IL-10/IL-1β ratios in resistant mouse compared
299 to susceptible hamster. As a limitation, evaluation of gene expression at the transcript level
300 might not correlate with protein regulation due to possible post-transcriptional regulation
301 mechanisms. Thus, we performed quantification of cytokine concentration in blood of LiVV-
302 infected mice and confirmed the increase of IL-10 levels in this resistant mouse model.
303 Assessment of cytokine concentration in hamster blood would also be of interest but specific
304 antibodies are hardly available for hamsters.

305 Interleukin-10 plays a major role in the resolution of inflammation during sepsis and
306 infection avoiding appearance of deleterious lesions but is also involved in persistence of
307 pathogens by interfering with innate and adaptive immunity. Indeed, this molecule acts on
308 several types of leucocytes including antigen-presenting cells (APCs), by downregulating
309 expression of co-stimulatory markers and production of proinflammatory cytokines [19]. It also
310 directly inhibits T-cell response and proliferation while down- regulating expression of Th1
311 type cytokines, IL-2 and interferon-γ (IFN-γ) [20]. Interestingly, in an IL-10 deficient murine
312 model of Lyme disease, sustained IFN-γ production was observed resulting in development of
313 disease related arthritis [21]. In parallel, IL-10 also has the potential to stimulate B cells
314 contributing to immunoglobulin class switching in these leucocytes [22]. Considering that IL-

315 10 is a multipotent anti-inflammatory cytokine with different facets closely associated with
316 susceptibility or resistance to infections and microbial persistence, we investigated the role of
317 IL-10 in leptospirosis pathogenesis using a pre-neutralization strategy by injecting anti-IL-10
318 antibodies in LiVV-infected mouse. Although not lethal, IL-10 pre-neutralization in
319 *Leptospira*-infected mice led to the development of a disease in this usually resistant model
320 with observations of clinical signs and weight loss during onset of the disease.

321 To better assess the role of IL-10 in the regulation of immune response occurring during
322 leptospirosis, effect of IL-10 previous neutralization was also evaluated on the regulation on
323 *Leptospira*-dependent cytokine gene expression. Surprisingly, no effect of IL-10 neutralization
324 was observed on LiVV-induced TNF-α transcript level. Experimental infection of C57BL/6
325 mice deficient for TNF-α receptor (TNFR) injected with pathogenic *L. interrogans* serovar
326 Copenhageni exhibited more severe renal lesions suggesting this cytokine as an important
327 mediators in the early control of infection [23]. Similarly, no difference was observed in TNF-
328 α expression level between resistant mouse and susceptible hamster during *Leptospira* infection
329 (Figure 3A). Contrasting with TNF-α results, IL-10 neutralization seemed to potentiate IL-1β
330 overexpression in LiVV-infected mice as observed for LiVV-infected susceptible hamsters.
331 Previous studies highlighted importance of pro-inflammatory cytokine IL-1β through
332 overstimulation of the inflammasome system that could contribute to tissue damages [24].
333 Leptospiral glycolipoprotein was also shown to activate the inflammasome system through
334 modulation of potassium transport and, together with *Leptospira* lipopolysaccharide, contribute
335 to the IL-1β overexpression and production induced by *L. interrogans* infection in susceptible
336 murine models [25]. Similarly, high IL-1β expression was associated with fatal outcome from
337 pulmonary haemorrhages in canine leptospirosis [26]. Considering that IL-1β overexpression
338 might be related to susceptibility and organ failures in *Leptospira*-infected host, the immuno-

339 protective role of IL-10 might be effective by regulating inflammatory response during
340 leptospirosis counterbalancing deleterious activities of pro-inflammatory IL-1β.

341 Effect of IL-10 neutralization was also evaluated on bacterial clearance and leptospiral
342 burden in blood and kidneys from LiVV-infected mice. Although no impact was observed in
343 leptospiremia, results showed evidences of IL-10 role in bacterial clearance in renal tissues.
344 Indeed, neutralization of IL-10 in *Leptospira*-infected mice led to a decrease in bacterial burden
345 in kidneys at D7 postinfection. Interestingly, contribution of IL-10 in the attenuation of the
346 inflammatory lesions during Lyme disease was shown but, interestingly, inhibition of
347 endogenous IL-10 also enhanced bacterial elimination [27]. Thus, IL-10 might be a major
348 component in the maintenance of *Leptospira* in the kidneys of reservoir mouse in its natural
349 environment.

350 This study suggests that the regulation of cytokine gene expression, especially the anti-
351 inflammatory IL-10, could be of importance in the leptospirosis pathogenesis. Indeed, lower
352 levels of IL-10 were shown in the blood of hamsters lethally infected regardless of the
353 *Leptospira* species used for inoculation. Moreover, inhibition of endogenous IL-10 in
354 *Leptospira*-infected mouse presented evidences of the dual role of this cytokine by regulating
355 immune response occurring during leptospirosis but exerting inhibitory effect on effective
356 bacterial clearance.

357

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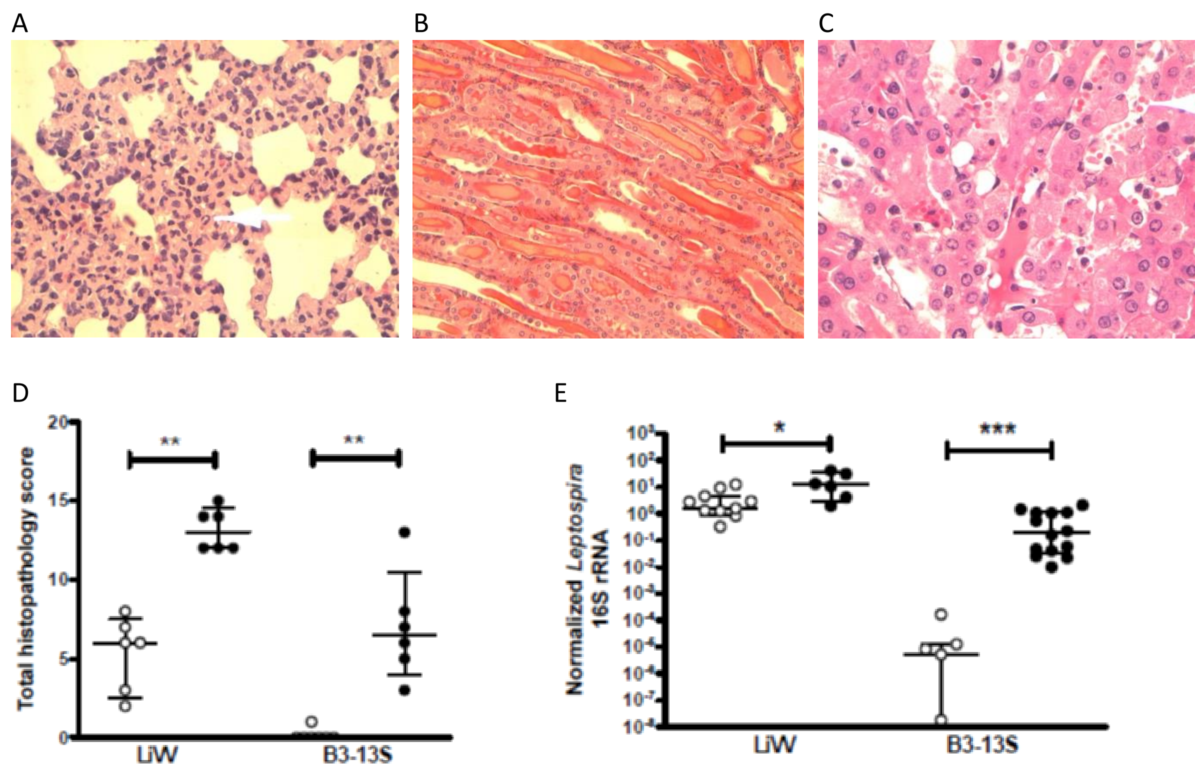
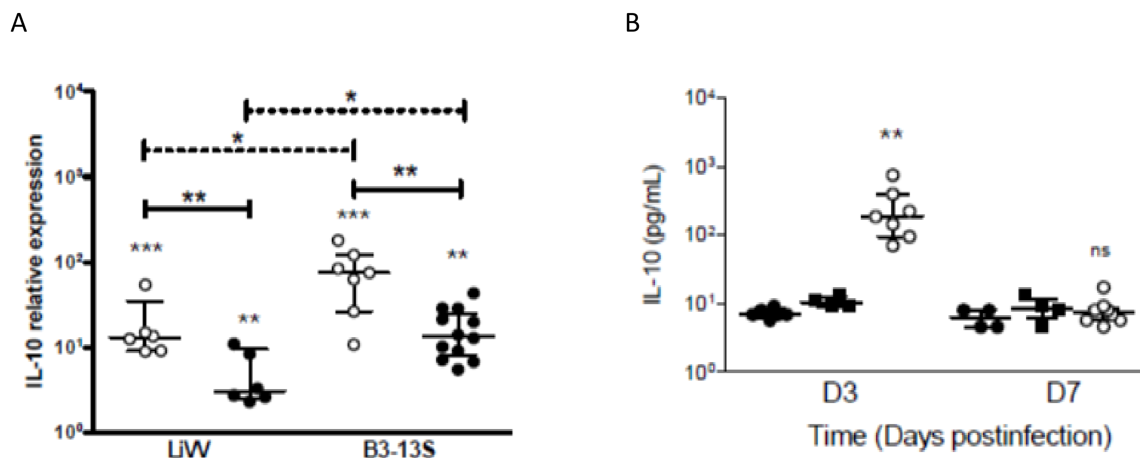
437

438 **Figures and captions**

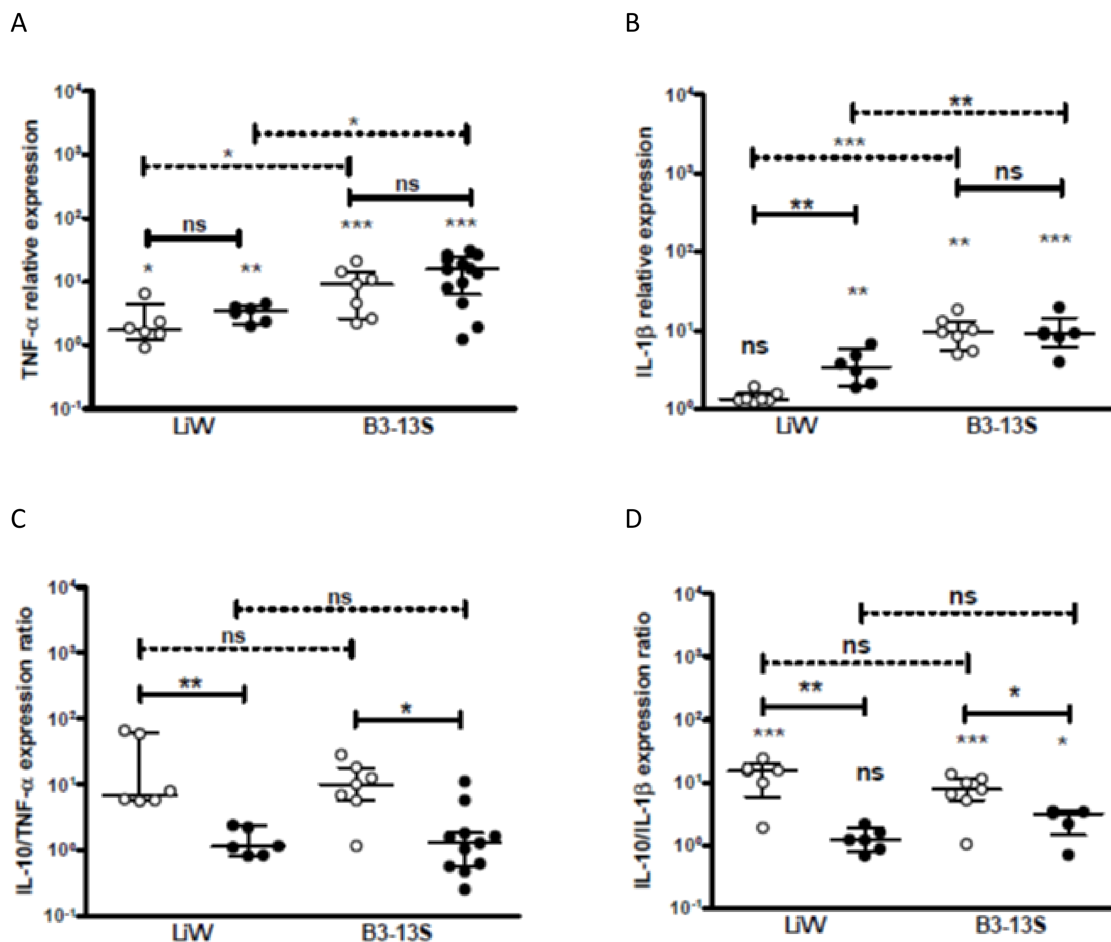
439



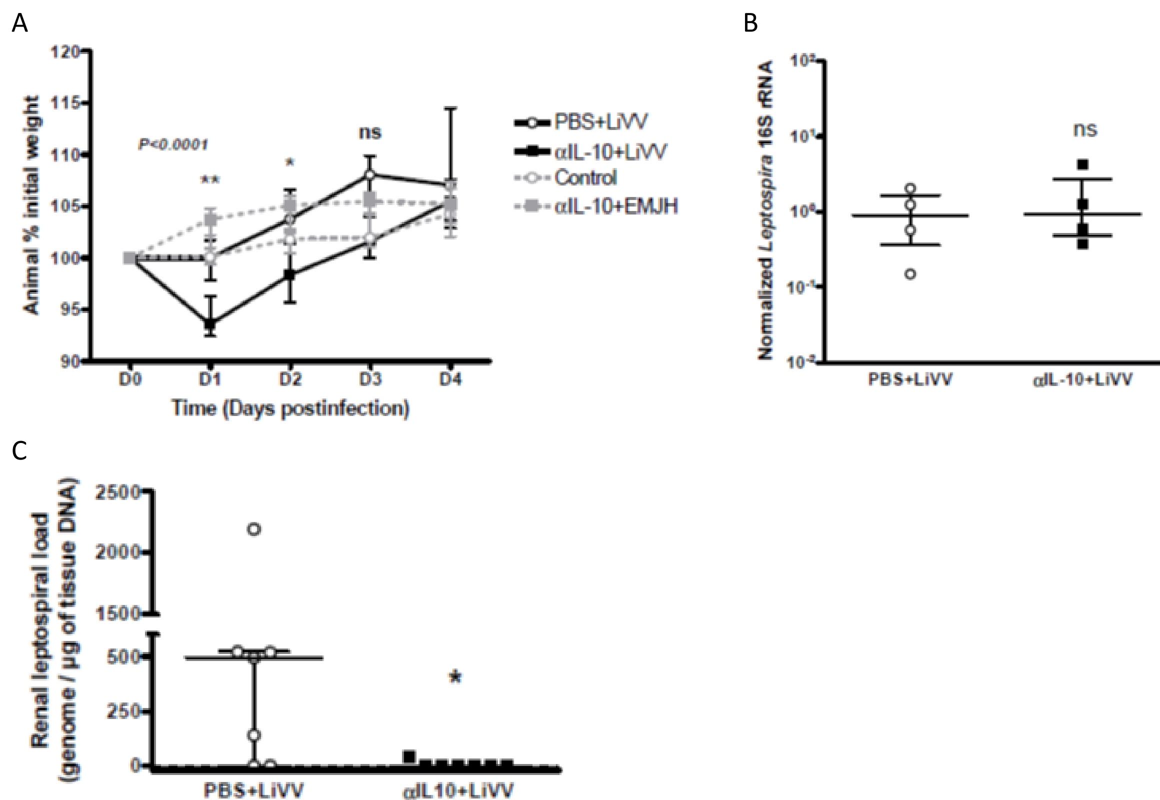
440 **Figure 1. Characterization of leptospirosis infection in resistant and susceptible animal models**
441 **injected with virulent *Leptospira* species.** Microscopic observations of tissues lesions in B3-13S
442 infected hamster were observed with intense congestion in all organs coming along with infiltration of
443 polymorphonuclear cells in lungs (A, open arrow), diffuse hemorrhages in renal tubules (B), and
444 sinusoidal dilatation in liver (C, open arrow) at D4 postinfection (HE, x400). Results present also
445 histopathological score in organs (D), and quantification of *Leptospira* 16S rRNA in blood (E) from mice
446 (open circles) and hamsters (filled circles) at D3-4 after infection with virulent *L. interrogans* serovar
447 Icterohaemorrhagiae strain Verdun (LiVV) or *L. borgpetersenii* serovar Ballum isolate B3-13S (B3-13S)
448 as described in Methods. Shown is the median (horizontal bars) ± interquartile ranges and individual
449 data (dots). Significant difference between animals was evaluated using a non-parametric Mann-
450 Whitney test. *P<0.05, **P<0.001, ***P<0.0005, ns: not significant.



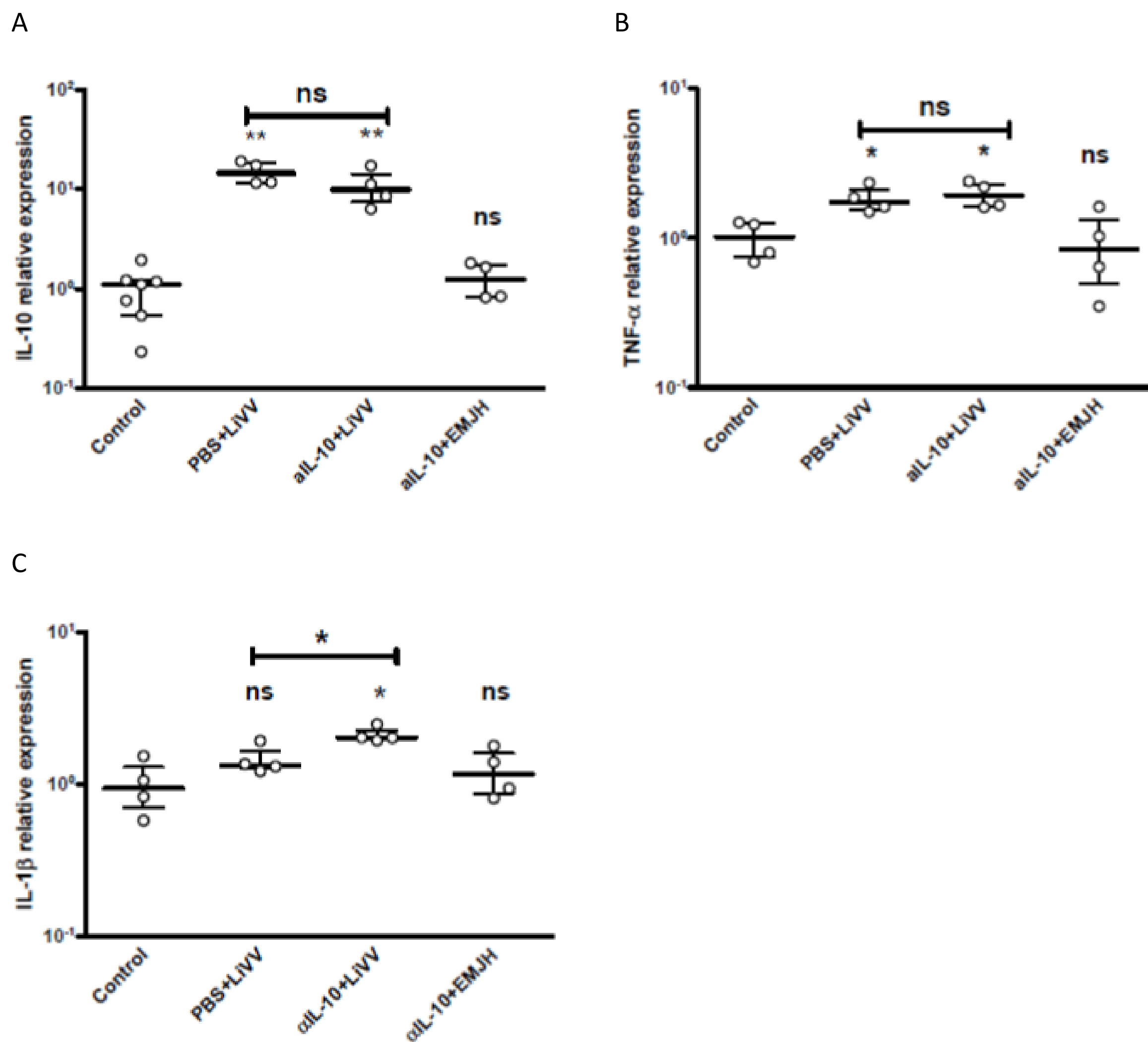
451 **Figure 2. Differential overexpression of IL-10 in blood of animals injected with virulent *Leptospira***
452 **species.** Results present relative gene expression of IL-10 (A) in blood from mice (open circles) and
453 hamsters (filled circles) at D3-4 after infection with virulent *L. interrogans* serovar Icterohaemorrhagiae
454 strain Verdun (LiVV) or *L. borgpetersenii* serovar Ballum isolate B3-13S (B3-13S). Protein level of IL-10
455 (B) was quantified in blood from mice infected with LiVV (open circles) compared to uninfected (filled
456 circles) or EMJH-injected (filled squares) control animals. Shown is the median (horizontal bars) ±
457 interquartile range and individual data (dots). Significant difference was evaluated compared to basal
458 level in uninfected animals (above asterisks), between animal models for each strain (solid lines) or
459 between strains for each animal model (dotted lines) using a non-parametric Mann-Whitney test.
460 **P*<0.05, ***P*<0.001, ****P*<0.0005, ns: not significant.



461 **Figure 3. Regulation of TNF-α and IL-1β gene expression in blood of animals injected with virulent**
462 ***Leptospira* species.** Results present relative gene expression of TNF-α (A) and IL-1β (B), and IL-10 to
463 TNF-α (C) and IL-10 to IL-1β (D) expression ratio in blood from mice (open circles) and hamsters (filled
464 circles) at D3-4 after infection with virulent *L. interrogans* serovar Icterohaemorrhagiae strain Verdun
465 (LiVV) or *L. borgpetersenii* serovar Ballum isolate B3-13S (B3-13S). Shown is the median (horizontal
466 bars) ± interquartile range and individual data (dots). Significant difference was evaluated compared
467 to basal level in uninfected animals (above asterisks), between animal models for each strain (solid
468 lines) or between strains for each animal model (dotted lines) using a non-parametric Mann-Whitney
469 test. *P<0.05, **P<0.001, ***P<0.0005, ns: not significant.



470 **Figure 4. Characterization of leptospirosis infection in mouse challenging IL-10 neutralization.** Body
471 weight (A) was monitored in mice (n = 7 or 8 individuals) until 4 days after infection with virulent *L.*
472 *interrogans* serovar Icterohaemorrhagiae strain Verdun (LiVV) and pre-injected with 200 µL of PBS
473 (PBS+LiVV) or 200 µg / 200 µL of anti-IL-10 antibody (αIL-10+LiVV) as described in Methods. Growth
474 curves of control mice without injection (Control) or double injected with anti-IL-10 antibody and EMJH
475 medium are also presented (αIL-10+EMJH). Values represent mean ± SD. Significant difference
476 between animals was evaluated using a 2-WAY ANOVA test to compare difference between PBS+LiVV
477 and αIL-10+LiVV for all the time challenge (*P< 0.0001*). Significant difference between treatments at
478 each time point was also evaluated using a non-parametric Mann-Whitney test. *P<0.05, **P<0.001,
479 ns: not significant. Quantification of *Leptospira* 16S rRNA in blood at D3 postinfection (B) and bacterial
480 load in kidneys at D7 postinfection (C) were measured in LiVV-infected mice pre-injected with 200 µL
481 of PBS (PBS+LiVV) or with 200 ng / 200 µL of anti-IL-10 antibody (αIL-10+LiVV). Shown is the median
482 (horizontal bars) ± interquartile ranges and individual data (dots). Significant difference between
483 conditions was evaluated using a non-parametric Mann-Whitney test. *P<0.05, ns: not significant.



484 **Figure 5. Regulation of cytokine gene expression in blood of LiVV-infected mice challenging IL-10**
485 **neutralization.** Results present relative gene expression of IL-10 (A), TNF-α (B) and IL-1β (C) in blood
486 from LiVV-infected mice after pre-injection with 200 µL of PBS (PBS+LiVV) or 200 µg / 200 µL of anti-
487 IL-10 antibody (αIL-10+LiVV) at D3-4 postinfection. Blood expression of cytokines in control mice
488 without injection (Control) or double-injected with antibody and EMJH medium (αIL-10+EMJH) were
489 shown. Shown is the median (horizontal bars) ± interquartile range and individual data (dots).
490 Significant difference was evaluated compared to basal level in uninfected animals (above asterisks)
491 or between treatments (solid lines) using a non-parametric Mann-Whitney test. *P<0.05, **P<0.001,
492 ns: not significant.

493

494 **Table 1. Details and sequence of primers used for qPCR assays**

Gene	GenBank [a]	Sequence (5'-3') [b]	Size [c]	Tm [c]	Efficiency [d]
Hamster β-actin	AF046210	(F) TCTACAACGAGCTGCG (R) CAATTTCCCTCTCGGC	357	88.33	1.802±0.04
Murine β-actin	NM_007393	(F) AAGAGAAGCTGTGCTATGTT (R) GTTGGCATAGAGGTCTTTACG	251	86.25	1.660±0.09
Hamster GAPDH	DQ403055	(F) CCGAGTATGTTGTGGAGTCTA (R) GCTGACAATCTTGAGGGA	170	85.67	1.938±0.04
Murine GAPDH	NM_008084	(F) TCATCCCAGAGCTGAACG (R) GGGAGTTGCTGTTGAAGTC	213	86.39	1.853±0.02
Hamster IL-10	AF046210	(F) TGGACAACATACTACTCACTG (R) GATGTCAAATTCATTCATGGC	308	85.50	1.871±0.05
Murine IL-10	NM_010548	(F) ATTCCCTGGGTGAGAAG (R) CTCTGTCTAGGTCCTGG	259	83.45	1.891±0.04
Hamster IL-1β	AB028497.1	(F) ATCTTCTGTGACTCCTGG (R) GGTTTATGTTCTGTCCGT	156	85.29	1.852±0.04
Murine IL-1β	NM_008361	(F) GTGTGGATCCCAAGCAATAC (R) GTTGTTTCCCAGGAAGACAG	161	83.35	1.660±0.09
Hamster TNF-α	AF046215	(F) AACGGCATGTCTCTCAA (R) AGTCGGTCACCTTTCT	278	88.05	1.849±0.03
Murine TNF-α	NM_013693	(F) CAACGGCATGGATCTCA (R) GGACTCCGCAAAGTCT	325	87.80	1.832±0.04
Leptospira 16S rRNA [e]	-	(F) GGCGGCGCGTCTTAAACATG (R) TTCCCCCCATTGAGCAAGATT	331	86.50	1.849±0.01
Leptospira lfb1 [f]	LA0322	(F) CATTCATGTTTCGAATCATTTCAAA (R) GGCCCAAGTTCCTTCTAAAAG	331	83.40	1.735±0.03
Leptospira LipL32 [g]	-	(F) AAGCATTACCGCTTGTGGTG (R) GAACTCCCATTTCAGCGATT (P) AAAGCCAG GACAAGCGCCG	242	-	1.788±0.03

495

496 [a] Accession Number of mRNA sequence in GenBank (NCBI) used for primer design.

497 [b] (F), (R) and (P) indicate forward and reverse primer and probe sequences, respectively.

498 [c] PCR product size in base pairs and melting temperature (Tm) in °C.

499 [d] Efficiency for PCR was determined by elaboration of standard curves as described in Material and

500 Method.

501 [e, f, g] See Mérien et al. 1995 [28], Mérien et al. 2005 [14] and Stoddard et al.[15], respectively.

502