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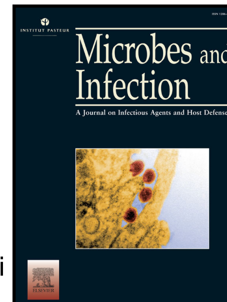

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Pathogenesis and immune response in *Brucella* infection acquired by the respiratory route

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Abstract

Brucella infection is frequently acquired through the respiratory route. The pathogen disseminates systemically from the lungs to infect peripheral organs. In this review we summarize the existing data on the pathogenesis of inhalational *Brucella* infection, the pulmonary immune response to the pathogen, and potential strategies for inducing protective lung immunity.

Keywords: *Brucella*; inhalational infection; pathogenesis; immune response; vaccines

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1. Clinical aspects and transmission of brucellosis

Brucellosis is an infectious disease caused by Gram-negative, facultative intracellular bacteria of the genus *Brucella*, that affects several species of domestic animals, wildlife and humans, with a significant impact on public health. *Brucella* invades, replicates and survives efficiently in phagocytic and several non-phagocytic cells causing chronic disease [1–3]. Brucellosis is the most common zoonosis, with more than 500,000 new reported human cases annually, and has a worldwide distribution, mainly affecting the Mediterranean countries, Central Asia, India, Arabic Peninsula, and Central and Latin America [4,5]. Brucellosis is a debilitating but rarely fatal disease. Acute human disease is characterized by non-pathognomonic clinical findings such as undulant fever, night sweats, splenomegaly, weight loss, myalgia, arthralgia and depression. Chronic disease can cause more severe complications such as osteoarticular brucellosis, neurobrucellosis and endocarditis, the latter being the main cause of the occasional fatal cases [6]. *B. melitensis*, *B. suis* and *B. abortus* are the most pathogenic species for humans and each one has a domestic animal as preferential host (small ruminants, swine, and bovines, respectively). In domestic animals, brucellosis causes reproductive diseases characterized by abortions, stillbirth, orchitis, epididymitis and infertility, causing severe economic losses in animal industry. It should be noted that there are no vaccines for human brucellosis, so the prevention of infection in humans depends almost exclusively on the control of infection in domestic animals through vaccination and other sanitary measures.

Brucella spp. usually enters its hosts through the mucosa. Human infection associated with consumption of unpasteurized dairy products has been widely documented [7,8], and there are also reports of contagion by contact of contaminated

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50 material with the ocular conjunctiva [9,10]. Inhalation of infected aerosols is a
51 frequent way to acquire the infection in humans. Outbreaks of human brucellosis
52 linked to airborne transmission have been reported in slaughterhouses, laboratories
53 producing *Brucella* vaccines, and rural areas [11–14]. Brucellosis is considered the
54 most common laboratory-acquired infection worldwide [15,16], and airborne
55 transmission have been implicated in most cases. Mucosal entry is also the main
56 form of infection among susceptible animals. In particular, the animals' habit of
57 sniffing and licking the placental and fetal remains from abortions, which in case of
58 coming from a *Brucella* abortion are contaminated with a very high load of bacteria,
59 contributes significantly to the spread of infection in the herds. In some species,
60 particularly goats, swine and dogs, spread through the venereal route is also
61 important.

62 Human brucellosis can be easily acquired by air transmission and therefore
63 *Brucella* can be considered a possible biological weapon. *B. suis* was the first agent
64 weaponized by the United States, in the 1950s [4,17]. It has been estimated that as
65 few as 10 to 100 aerosolized organisms are required to generate disease in humans.
66 The high infective capacity of *Brucella* when delivered in this manner, its ability to
67 spread easily, and the chronic and debilitating nature of human disease has led to
68 the Centers for Disease Control and Prevention and the National Institute of Allergy
69 and Infectious Diseases to classify *B. melitensis, B. abortus*, and *B. suis* as Category
70 B bioterrorism agents [4,17].

71 Although *Brucella* spp. enters the body very frequently through the respiratory
72 tract, most studies on *Brucella* pathogenesis and immunity have been conducted in
73 animal models of intraperitoneal infection and to a lesser extent in models of oral
74 infections. However, in the last decade some studies have begun to elucidate the

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75 host-pathogen interaction during *Brucella* respiratory infection. In this review we will
76 discuss the existing data on the pathogenesis of *Brucella* infection acquired through
77 the respiratory route, the pulmonary immune mechanisms against such infection, and
78 potential strategies for inducing protective lung immunity.
79

80 ## 2. Pulmonary brucellosis in humans

81 Respiratory manifestations are relatively infrequent in human brucellosis, even in
82 patients with documented or strongly presumed airborne infection. A distinction must
83 be made between pulmonary involvement in cases of airborne transmission and that
84 occurring in patients with brucellosis acquired through other infection routes. In the
85 first case, the pathogen reaches the lungs from the alveolar space and establishes
86 early interactions with alveolar epithelial cells and macrophages, which are the first
87 cells involved in the local immune response to this infection. From this location the
88 bacterium disseminates systemically to establish infection in peripheral organs. In the
89 second scenario, in contrast, the pathogen has previously interacted with the
90 systemic immune effectors and has probably established infection in other organs
91 before reaching the lung through hematogenous dissemination. It may be speculated
92 that these differences between the infection routes may eventually translate into
93 differences in the pathological phenomena taking place in the lung during pulmonary
94 brucellosis.

95 As mentioned, airborne transmission of *Brucella* has been linked to human
96 cases in slaughterhouses, clinical microbiology laboratories, vaccine production
97 plants, and rural areas [11–16]. A large study by Kauffmann et al. analyzed the data
98 from 6 brucellosis outbreaks (387 cases) occurred in the 1960-1976 period in
99 abattoirs in USA and clearly established the airborne nature of the disease, but

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100 clinical manifestations were not described [12]. Other studies on airborne brucellosis
101 report clinical data and a few provide information on respiratory involvement. Typical
102 manifestations of brucellosis (fever, myalgia, adenopathies, etc.) were found in 8
103 patients presumably infected through aerosols in a clinical microbiology laboratory,
104 but pneumonitis was detected in only one of them (12%) [13]. In a review of 60 cases
105 of laboratory-acquired brucellosis registered in the USA from 1945 to 1957, 21 of
106 which occurred after documented laboratory accidents, cough was found in 33% and
107 pulmonary rales in 8.3% [18]. Unfortunately, no details were provided about the
108 nature of the accidents to establish the likelihood of airborne transmission. An
109 outbreak of airborne infection in a laboratory producing *B. melitensis* Rev-1 vaccine
110 involved 22 symptomatic patients, most of which presented the typical brucellosis
111 manifestations. Of note, however, 6 of them presented epistaxis [19]. Airborne
112 transmission was considered the most likely route of infection for 33 rural workers
113 infected with *B. melitensis* in Argentina, from which 9.1% had pneumonitis and
114 bronchitis [14]. Only general brucellosis findings were reported in other cases of
115 probable airborne brucellosis, including 4 patients from a clinical microbiology
116 laboratory [20], 3 workers that got the infection from sniffing *Brucella* cultures [21],
117 and 12 employees from a laboratory in which a flask containing a *Brucella* culture
118 was accidentally broken [22]. A review of laboratory-acquired brucellosis cases
119 reported in the literature from 1982 to 2007 identified 59 cases linked to aerosol
120 exposure (83% of 71 total cases) [16]. The study also analyzed separately 121 cases
121 of airborne brucellosis previously reported in summary reports. In both groups of
122 patients, the most frequent clinical findings were fever, arthralgia, sweats, headache,
123 myalgia and malaise. No pulmonary findings were reported.

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124 Other case reports and reviews have described the clinical and pathological
125 findings in brucellosis cases exhibiting pulmonary involvement, regardless of the
126 route of infection. Moreover, in most of these cases the route of infection is unknown.
127 The most recent review on this subject, performed by Solera and Solís García del
128 Pozo [23], has collected data from case reports and also from three major previous
129 reviews performed by Pappas et al. [24], Hatipoglu et al. [25] and Erdem et al. [26].
130 The study only included brucellosis patients with respiratory involvement confirmed
131 by radiography or computed tomography (n= 253). Cough was present in about 64%
132 of the patients from case reports, and in 45-86% of patients in the three main
133 previous reviews. Expectoration was reported by around 32% and 27-32%,
134 respectively, and dyspnea was reported by 21% and 21-61%. Chest pain was
135 present in 33% of the patients in case reports but was much less frequent in the
136 previous reviews. In the case reports the most frequent radiological manifestation
137 was pleural effusion (47.2%), followed by pneumonia (41.7%), pulmonary nodules
138 (19.4%), interstitial pattern (18.1%) and mediastinal or thoracic lymph nodes (9.7%).
139 The main radiological pattern found in the three previous reviews varied from
140 pneumonia (68.4%) [26] to interstitial pattern (40.5%) [24] or pulmonary nodules
141 (48.6%) [25]. In the few cases reporting histopathological data, granulomatous
142 lesions were described.

143

144 3. Course of respiratory *Brucella* infection in animal models

145 The fact that the respiratory mucosa serves as a natural site of entry of *Brucella* to
146 the host, and the potential use of this bacterium as a biological weapon agent, has
147 led to the development of animal models of respiratory infection to evaluate the
148 efficacy of novel vaccines and treatments for brucellosis. The mouse has been the

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149 most extensively animal model to asses these topics due to its easy handling in
150 laboratories despite not being a natural host. Intranasal (i.n.), intratracheal (i.t.),
151 nose-only and whole-body aerosol routes of challenge have been used to establish
152 brucellosis in Balb/c mice [27–32]. In all the inoculation routes studied, *Brucella* could
153 rapidly disseminate from the site of challenge to the spleen and liver.

154 *B. abortus* infection of mice via the whole-body aerosol route results in rapid
155 colonization of lung tissue that is sustained or increases during the first weeks post-
156 challenge and then gradually decreases over time, indicating the ability of this
157 pathogen to replicate within the lung [31]. A similar behavior was observed when *B.*
158 *suis* was used for challenge [30,33]. In contrast, *B. melitensis* 16M inoculated by
159 different routes colonizes the lungs but does not replicate in these organs, and the
160 count of viable bacteria remains constant or decreases over time depending on the
161 doses received [27,30–32]. In addition, viable bacteria are detected in the lungs at
162 prolonged times after infection (8 weeks post-challenge) [31,32]. This suggests that
163 the lung is a persistence niche for *Brucella* in the host. Surprisingly, no significant
164 histological changes are observed in the lungs of *B. abortus*- or *B. melitensis*-infected
165 mice [27,28]. Henning et. al. described perivascular or peribronchiolar mononuclear
166 cell infiltration in only 17% of the animals infected by the aerosol route at the highest
167 dose tested [32]. The limited inflammatory immune response to *Brucella* in the lungs
168 may be due in part to the ability of the pathogen to actively modulate the pulmonary
169 innate immune response as described by Hielpos et al. [28] (see below).

170 *B. abortus*, *B. melitensis* and *B. suis* administered by different routes of
171 exposure (i.n., i.t., nose-only and whole-body aerosol routes) can be found in the
172 spleen of infected animals in the first or second week post-challenge depending on
173 the time tested and the doses used [27–33]. In contrast to what occurs in the lung,

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174 the load of *B. melitensis* in the spleen increases until week 4 post-challenge and then
175 decreases [31]. Similar results were described for *B. suis* [30,33]. Conversely, the
176 burden of *B. abortus* in the spleen increases steadily over time until the end of the
177 experiments (8 weeks post-challenge) [31]. As mentioned, splenomegaly is a
178 common clinical manifestation of human brucellosis. Splenomegaly was evident in
179 animals infected by the i.n. route or whole-body aerosol routes with *B. melitensis* at 3
180 or 4 weeks after challenge, respectively [27,31]. Histological evaluation of the
181 spleens of these animals showed an increase in the white pulp and the marginal
182 zone [27]. Whole-body aerosol infection with *B. abortus* also generated
183 splenomegaly but this was evident later (6 weeks post-challenge) [31].

184 The liver is another target organ during *Brucella* respiratory infection. *B.*
185 *abortus*, *B. melitensis* and *B. suis* was detected in liver during the first weeks after
186 challenge and increased over time [27,30,31,33]. Consistently with human disease,
187 *B. melitensis* respiratory infection causes inflammation in the liver [27,32]. It is still
188 unknown whether respiratory infection with *B. abortus* or *B. suis* causes histological
189 lesions in this organ. Notably, Smither et al. have described that *B. suis* and *B.*
190 *melitensis* also have tropism for the uterus in mice challenged by aerosolization [30].
191 These results are consistent with the detection of bacteria in the reproductive
192 systems of ruminants and in the uterus of other naturally infected animals, such as,
193 otters and seals [34]. Although fever is one of the most common symptoms of human
194 disease, increases in body temperature have not been detected during nose-only
195 aerosol infection with *B. melitensis* [32]. In concordance to human disease, bacteria
196 were isolated from the blood of some infected mice. Positive blood cultures were
197 variable, reaching 62% of infected mice at the highest dose tested. These positive
198 cultures were observed only after blood samples were enriched prior to plating [32].

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199 In the same report, endocarditis was observed in 8% of challenged mice, which is
200 consistent with this rare complication being observed in human patients.
201 Non-human primates have also been used as models for *Brucella* infection
202 due to their similar susceptibility to infectious diseases as compared to humans.
203 Several studies demonstrated that rhesus macaques are susceptible to aerosolized
204 *B. melitensis* infection, as demonstrated by systemic dissemination from the
205 challenge sites and histology [35–37]. This animal model has not been used to
206 assess susceptibility to respiratory infection by *B. abortus,* and only one study
207 analyzed tissue burden for the first week after aerosol exposure with a high dose of
208 *B. suis* 1330 [38]. In contrast to what occurs in the murine model, rhesus macaques
209 that received an aerosol challenge with *B. melitensis* or *B. suis* developed undulating
210 fever [37,38]. The bacteria quickly spread from the challenge site to the liver, spleen
211 and kidneys, among other tissues [35–37]. The burden of *B. melitensis* in the lung,
212 liver, kidneys and spleen was greatest on day 14 post-challenge and decreased over
213 time. At the end of the study (day 56 post-challenge) bacteria were still detected in
214 the organs, although at very low values [37]. However, it is still unknown whether
215 rhesus macaques develop sterilizing immunity or if they are chronically infected with
216 a few bacteria. Positive blood cultures were observed after challenge by *B. melitensis*
217 in 50% of infected animals [35,37]. Histopathologic examination revealed lesions
218 attributed to *Brucella* infection in the liver, kidneys, lymph nodes, lungs, and/or spleen
219 of all animals [35–38]. Splenomegaly was reported in all studies of aerosolized *B.*
220 *melitensis*. Mense et al. demonstrated the presence of *B. melitensis* and
221 inflammatory lesions in the testes and epididymis of some infected macaques [35],
222 which is similar to human brucellosis, in which the *Brucella* location in the male
223 reproductive tract is observed in approximately 2% to 10% of reported cases. In other

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224 study, *B. melitensis* was cultured from the saliva and vaginal vault of infected
225 animals, demonstrating bacterial dissemination to other target tissues [39]. Infection
226 with aerosolized *B. melitensis* only generates changes in some clinical laboratory
227 parameters, such as an increase in C-reactive protein and in certain liver enzymes,
228 which is consistent with what has been observed in human brucellosis [37].

229 Guinea pigs have been also used since the beginning of the 20th century to
230 assess the pathogenicity of respiratory infection with *B. suis* and *B. melitensis*. As in
231 the murine model, *B. suis* replicated in the lungs of guinea pigs infected by the
232 aerosol route [40,41]. Bacterial dissemination from lungs to peripheral organs only
233 occurred when the bacteria have reached the regional lymph nodes and blood [40].
234 The burden in the spleen increased from day 11 until day 28 post-challenge, and
235 then decreased. At the end of the study (215 days post-challenge) viable bacteria
236 were still detected in the spleen of some animals. Splenomegaly developed in all
237 infected animals and macroscopic lesions were observed in the spleen and the
238 bronchial and cervical lymph nodes. Macroscopic lesions were evident in the lungs
239 only after day 96 post-challenge. Recently, Hensel et al. demonstrated that *B.*
240 *melitensis,* inoculated in guinea pigs by the i.t. route in high doses (10^7-10^9),
241 colonizes the spleen, the uterus and the tracheobronchial and cervical lymph nodes
242 as early as 2 hours post-challenge [42]. *B. melitensis* does not replicate in the lung
243 and the number of bacteria decreases with time post-infection (p.i.). In contrast, the
244 bacterial burden in the liver, uterus, spleen, and cervical and tracheobronchial lymph
245 nodes increases over time. Notably, inoculation of a low dose of *B. melitensis* (10^1
246 and 10^2 CFU) in guinea pigs did not result in colonization of any tissue examined.
247 Animals infected with the highest dose developed fever, splenomegaly and
248 histological changes in the all tissue evaluated [42].

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249 In summary, the studies described demonstrate that guinea pigs and rhesus
250 macaques infected through the respiratory route develop characteristic signs and
251 symptoms of the disease that mimic human brucellosis and therefore support the use
252 of these animal models to assess the efficacy of new vaccines and therapies against
253 *Brucella* inhalational infection.

254 All these studies clearly demonstrate that *Brucella* can reach the bloodstream
255 and peripheral organs from its initial site of entry in the lungs. The mechanisms used
256 by the pathogen to cross the lung epithelial barrier and gain access to the blood
257 and/or lymphatic circulation in order to disseminate have not been clarified.
258 Respiratory pathogens have evolved many strategies to interfere with cell-cell
259 junctions, increase epithelial permeability, destabilize epithelial structure and
260 function, and sometimes cross and/or break the barrier that constitutes the epithelium
261 [43]. It has been shown that *Brucella* spp. can adhere and invade human bronchial
262 and alveolar epithelial cells [44]. While rough strains (*Brucella canis* and *Brucella*
263 *abortus* RB51) are internalized more efficiently than smooth strains (*B. abortus* 2308
264 and *Brucella suis* 1330), only the latter replicate intracellularly. The expression of the
265 type IV secretion system (T4SS) encoded by *virB* genes is essential for the
266 intracellular replication of *Brucella* in lung epithelial cells. However, this infection does
267 not seem to induce significant respiratory epithelial cells death.

268 For some infections by airborne bacteria that can survive inside macrophages,
269 it has been postulated that infected alveolar macrophages (AM) could migrate to the
270 systemic circulation carrying viable pathogens that can later establish infection at
271 distant sites, thus constituting a Trojan horse mechanism [45]. Of note, a study by
272 Archambaud et al. in mice infected with *B. abortus* through the intranasal route
273 showed that AM harboring live brucellae migrate within a few days p.i. to the lung-

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274 draining mediastinal lymph nodes where intracellular replication of the pathogen
275 takes place [46]. Therefore, this study suggested that *Brucella* can replicate
276 intracellularly in AM and that these cells can act as Trojan horses for bacterial
277 dissemination. Later in vitro studies confirmed the ability of smooth *Brucella* species
278 to survive and replicate in murine AM and porcine AM [47,48]. However, the survival
279 and replication of brucellae in AM seems to vary with the *Brucella* species and the
280 host species. While *B. suis* was able to invade and replicate in AM from hooded
281 seals (*Cystophora cristata),* different strains of *B. ceti* and *B. pinnipedialis* were able
282 to invade but not to establish a persistent infection in these cells [49].
283

284 **4. Immune response to respiratory *Brucella* infection**

285 **4.1. Innate immune response**

286 Once inhaled, *Brucella* microorganisms may interact with the respiratory epithelium,
287 the AM and, later, the underlying fibroblasts. All these cell types have immunological
288 relevance, due to their ability to internalize bacteria and, eventually, produce
289 antigenic presentation, and/or due to their ability to produce mediators (cytokines,
290 chemokines, antimicrobial peptides, etc.) in response to bacterial antigens and/or to
291 cytokines produced by other cells [50].

292 In addition to its function as a physical barrier between the airway lumen and
293 blood circulation, the airway epithelium also displays immunological activities. Human
294 bronchial epithelial cells secrete IL-8, MCP-1, CCL20 and GM-CSF upon infection
295 with *B. abortus.* Alveolar epithelial cells do not secrete IL-8 or MCP-1 but secrete
296 CCL20 in response to the infection [51,52]. Notably, most of these responses are
297 also produced by *B. abortus* antigens. Bronchial epithelial cells secrete IL-8, CCL20
298 and GM-CSF after stimulation with heat-killed *B. abortus* (HKBA), cytoplasmic

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299 proteins and LPS from *B. abortus,* whereas alveolar epithelial cells secrete CCL20 in
300 response to a lipidated outer membrane protein from *B. abortus* (L-Omp19)
301 demonstrating that *Brucella* antigens can induce *per se* the secretion of chemokines
302 and growth factors by lung epithelia [51,52].

303 Human lung epithelial cells are known to secrete beta-defensins (hBD) with
304 antimicrobial properties, either constitutively (hBD1) or in response to infections with
305 respiratory pathogens. Human alveolar epithelial cells do not secrete hBD2 in
306 response to *B. abortus* infection, but secretion is induced in response to factors
307 secreted by *Brucella*-infected monocytes (IL-1β) [52] (see below). Nevertheless,
308 hBD2 and hBD3 have no bactericidal activity against *B. abortus* even at levels much
309 higher than those required to kill *Escherichia coli*.

310 In close contact with the alveolar epithelium are AM, the main phagocytic
311 immune cells in lung [53]. In response to *B. abortus* infection murine AM secret TNF-
312 α, KC (CXCL1, neutrophil chemoattractant), IL-1β, IL-6 and IL-12, albeit at lower
313 levels than peritoneal macrophages [47]. Studies using knockout (KO) mice for TLR
314 receptors revealed that TNF-α and KC responses are mediated by TLR2 recognition.
315 In contrast, a cell line of porcine AM does not seem to produce TNF-α in response to
316 *B. suis* infection, and this appears to be related to modulation by a bacterial outer
317 membrane protein (Omp25) [48]. This diminished TNF-α response correlates with an
318 enhanced survival of wild type *B. suis* in porcine AM as compared to a Δ*omp25*
319 mutant.

320 While the responses described above have been evaluated using single cell
321 types (either epithelial cells or AM), in the in vivo situation a crosstalk between lung
322 epithelial cells and AM or other macrophagic populations can take place and may be
323 an important step to mount an immune response after *Brucella* inhalation. *B. abortus*-

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324 infected cocultures of human bronchial or alveolar epithelial cells and monocytes
325 release IL-8 and/or MCP-1 in higher levels than those produced by the respective
326 monocultures [51]. Moreover, soluble factors secreted by one cell type can stimulate
327 the secretion of cytokines or chemokines by the other cells in the absence of direct
328 contact. In fact, conditioned medium from *B. abortus*-infected monocytes induces IL-
329 8 and MCP-1 secretion by lung epithelial cells, and this effect is mediated by TNF-α
330 or IL-1β. Reciprocally, conditioned medium from *Brucella*-infected bronchial epithelial
331 cells induces MCP-1 production by monocytes in a GM-CSF-dependent manner [51].
332 Similarly, it has been shown that the production of CCL20 by lung epithelial cells can
333 be enhanced, and that of hBD2 can be induced, by factors (namely, IL-1β) produced
334 by *Brucella*-infected monocytes [52].

335 As in most infectious diseases, TLR receptors are relevant for the immune
336 response to *Brucella* infection [54]. Studies using TLR KO mice have been central for
337 establishing the role of TLR in the response to airborne *Brucella* infection. According
338 to a study in TLR KO mice of C57BL/6 background, TLR2, TLR4 and the MyD88
339 adaptor molecule (which is involved in the signaling pathway of most TLR) do not
340 seem to contribute to the control of lung infection in the first two weeks after aerosol
341 exposure to *B. melitensis*. In contrast, the three molecules seem to have a role in the
342 control of pulmonary infection from week 4 onwards, suggesting a contribution via
343 their impact on adaptive immunity [55]. A similar study using TLR KO mice of BALB/c
344 background and intranasal *B. abortus* infection showed a clear trend to a reduced
345 control of lung infection at two weeks p.i. in TLR2 KO mice, although differences did
346 not reach statistical significance [56]. In contrast, the lung burdens in TLR4 and TLR9
347 KO mice were very similar to the wild type controls. In another study, bacterial counts
348 in AM and lung homogenates obtained at one week p.i. from mice intratracheally

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349 infected with *B. abortus* were significantly higher in TLR2 KO animals than in
350 C57BL/6 controls [47]. Therefore, despite some discrepancies probably related to
351 differences in the infection models used, the available studies suggest that TLR are
352 involved in the early and/or late control of pulmonary *Brucella* burden after respiratory
353 infection. The mechanisms by which TLR signaling contributes to the control of
354 pulmonary *Brucella* infection have not been established. Several studies suggest that
355 TLR are involved in the production of proinflammatory cytokines in the lungs of
356 *Brucella*-infected mice. However, mice infected intratracheally with *B. abortus* show a
357 limited inflammatory response in the lungs during the first week p.i., a phenomenon
358 related to the expression of bacterial proteins (BtpA and BtpB) that can modulate
359 TLR signaling [28]. Of note, the lungs from mice infected with a double mutant for Btp
360 proteins present a stronger inflammatory infiltrate than those infected with the wild
361 type strain of *B. abortus*, and the pulmonary levels of proinflammatory cytokines are
362 also higher in the former. This increased inflammation, however, did not reduce the
363 bacterial burden in the lungs of mice infected with the Btp mutant as compared to
364 those infected with the wild type strain [28]. In contrast, the expression of Btp
365 proteins conferred a survival advantage in the context of a stronger lung inflammation
366 induced by LPS from *E. coli*. Therefore, it may be possible that TLR-mediated
367 inflammation contributes to the control of pulmonary *Brucella* infection, but the level
368 of inflammation attained in the early stages of infection is not enough to produce this
369 effect. In addition, the contribution of TLR to the early control of pulmonary *Brucella*
370 infection mentioned above may operate by mechanisms alternative or
371 complementary to the induction of proinflammatory cytokines.

372 Besides TLR, other innate sensors may contribute to the recognition of
373 *Brucella* infection and the elicitation of immune responses in the lung.

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374 Inflammasomes are cytosolic multimeric complexes that mediate the cleavage of pro-
375 IL-1β and pro-IL-18 into their mature active forms [57]. Inflammasomes include
376 caspase-1 (which mediates the cleavage of pro-IL-1β) and a sensor component
377 (such as NLRP3, NLRC4, AIM2, etc.) responsible for detecting microbial components
378 (PAMPs) or cellular damage (DAMPs), and may also include an adaptor molecule
379 that connects the first two. Upon activation, inflammasomes mediate the proteolytic
380 cleavage of pro-IL-1β into mature IL-1β, which is the form of the cytokine that can be
381 secreted. IL-1β has a central role in the early pulmonary immune response to inhaled
382 pathogens, as it induces the expression of several chemokines and adhesion
383 molecules, enhances the phagocytic activity of neutrophils and monocytic cells, and
384 increases the production of reactive oxygen species [58]. Of note, IL-1β levels were
385 increased in the first days p.i. in lung homogenates and bronchoalveolar lavage fluid
386 (BALF) of mice intratracheally inoculated with *B. abortus*, but were comparatively
387 reduced in caspase-1 KO mice [59]. Interestingly, the pulmonary CFU numbers were
388 higher in mice lacking the IL-1 receptor (IL-1R) than in wild type mice, and the same
389 was true for mice lacking some inflammasome components (caspase-1, AIM2,
390 NLRP3). As mentioned, one of the protective functions of IL-1β is to induce the
391 expression of chemokines in lung cells. Notably, the levels of CXCL1 (KC) and the
392 number of neutrophils in BALF during the first days p.i. were significantly reduced in
393 caspase-1 KO mice as compared to controls. Therefore, this study shows that the
394 NLRP3 and AIM2 inflammasomes, probably through their ability to induce IL-1β
395 maturation, are involved in pulmonary innate immune protective mechanisms against
396 respiratory *B. abortus* infection. At variance with the protective role of IL-1R found in
397 this study, a study on intranasal *B. melitensis* infection did not find increased CFU
398 counts in the lungs of mice deficient for IL-1R, IL-6, TNF-α, or CCR2 [60]. This

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399 discrepancy regarding the role of IL-1R may relate to the differences between both
400 studies in the *Brucella* species, the infection route and the infecting dose.

401 Taken together, these data show that lung cells are susceptible to *Brucella*
402 invasion and intracellular replication. This pathogen exhibits numerous PAMPs that
403 can be recognized by innate immune receptors (TLR and inflammasomes) in airways
404 epithelial cells and AM. These cells secrete cytokines, chemokines and antimicrobial
405 peptides that would be expected to exert a rapid control of the infection. The efficacy
406 of these responses, however, is hampered by several characteristics and virulence
407 factors of the pathogen, including its ability to survive for long periods in infected
408 cells, its resistance to beta-defensins, and its capacity to modulate TLR-dependent
409 cytokine responses. Innate immune responses of lung cells to *Brucella* and the main
410 mechanisms used by the pathogen to evade such responses are summarized in
411 **Figure 1**.

412 **4.2. Adaptive immune response**

413 *Brucella* is able to evade the adaptive immune response allowing it to establish a
414 chronic infection. Although the mucosal immune system represents the first line of
415 defense against *Brucella* infection in nature, only very few studies have characterized
416 the adaptive immune response during respiratory infection. Hanot Mambres et al.
417 evaluated the immune response after primary and secondary i.n. infection of
418 C57BL/6 mice with virulent *B. melitensis* [60]. Using genetically deficient mice, they
419 demonstrated that TNF-α, MHC-II and IFN-ɣR deficiencies impair the late control in
420 the lungs after primary infection. In addition, IL-17RA deficiency was associated with
421 a higher bacterial burden in the lungs at day 5 p.i., a time at which IFN-ɣR deficiency
422 had no impact. In IFN-ɣR KO mice the bacterial burden on all organs tested
423 increases over time, and all animals die after 35 days p.i. These results demonstrate

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424 that functional IFN-ɣ is crucial for late control during primary infection. Primary
425 infection induces development of a protective memory that limits the dissemination of
426 bacteria from the lungs to the systemic organs after secondary infection. Only
427 deficiency in TCR-β affects the protective immune response against secondary
428 infection [60]. Notably, MHC-II or TAP-1 deficiency did not affect the efficiency of the
429 protective immune response, suggesting that both CD4+ and CD8+ α/β^+ T cells are
430 equally capable to mount a protective immune response against i.n. *Brucella*
431 infection. Although IL-12p35 deficiency did not affect the protective memory, IL-17A
432 neutralization in IL-12p35$^{-/-}$ mice affected the protection conferred against *Brucella*
433 challenge, which suggests that the reduced IFN-γ-mediated response can be
434 compensated for by an IL-17A-mediated response. This study demonstrates that
435 CD4+ T cells are essential for the development of a protective memory response
436 against i.n. secondary infection. In addition, CD8+ T cells can compensate for the
437 absence of CD4+ T cells to generate protection against i.n. *Brucella* infection.

438 Recently, a study showed that, in a mouse model of allergic asthma, the
439 development of the dominant IL-4 (Th2) immune response favors the growth of
440 *Brucella* in the lungs of infected animals [61]. This result confirms the relevance of
441 the Th1 immune response in the control of *Brucella* in the lung.

442 TLR activation is essential for the cellular adaptive immune response, as it
443 induces maturation of antigen presenting cells (APC), and improves antigen
444 presentation and cytokine production. The cytokine profiles produced by APC
445 determinate the differentiation of CD4+ T cells into Th1 or Th2 cells. In vivo studies
446 demonstrated that TLR2, TLR4 and MyD88 signaling are required for efficient
447 clearance of *Brucella* from lung following aerosol challenge [59]. Although not
448 experimentally demonstrated, deficiency in these TLRs is likely to affect

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449 differentiation of the cellular immune response to a Th1 profile required for efficient
450 control of *Brucella* from lung [38].

451 As mentioned, AM constitute the main cellular target of inhaled brucellae. Like
452 macrophages located in other tissues, AM processes microbial antigens and displays
453 antigenic peptides in the context of MHC molecules for recognition by specific T cells.
454 IFN-γ activates macrophages and induces their expression of MHC-II molecules,
455 resulting in enhanced antigen presentation to specific CD4+ T cells. Therefore,
456 pathogens that can induce downregulation of IFN-γ-induced MHC-II expression in
457 macrophages can hinder the recognition of infected cells by specific T lymphocytes,
458 thereby preventing some adaptive immune responses. Interestingly, in vitro studies
459 have demonstrated that *B. abortus* infection downregulates the expression of MHC-II
460 molecules induced by IFN-γ in AM from BALB/c and C57BL/6 mice [47]. The same
461 reduction was induced by HKBA or L-Omp19, and it was shown to be mediated by
462 TLR2 recognition. In addition, either L-Omp19 or HKBA reduced the antigen
463 presentation to T lymphocytes by AM [47]. Downmodulation of MHC-II expression by
464 *B. abortus* may contribute to its persistence for a long time in the lungs of infected
465 mice. The main adaptive immune responses involved in the control of *Brucella*
466 infection in the lung, and the evasion mechanism just described, are depicted in
467 **Figure 1**.

468 *Brucella* respiratory infection generates a specific humoral immune response. An
469 increase in specific antibodies has been observed in the murine and rhesus macaque
470 models following respiratory challenge [42,55,60]. Pei et al. demonstrated that TLR2
471 and TLR4 are required to generate early specific IgG, but not during the last stages
472 of infection (10 weeks post-challenge) in mice following aerosol exposure to *B.*
473 *melitensis* [55]. TLR2 and TLR4 do not participate in IgA secretion and are only

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474 required transiently for IgM production. In contrast, MyD88 is indispensable for the
475 production of specific IgG during all times tested. However, B cell deficiency does not
476 affect the bacterial burden in tissue during primary and secondary i.n. infection with
477 *B. melitensis*. This demonstrates that humoral immunity does not play a crucial role
478 in the control of i.n. *Brucella* infection in the mouse model [60].
479

480 **5. Vaccination against inhalational brucellosis**

481 Brucellosis can be naturally acquired by *Brucella* inhalation in both human and
482 animals, therefore, numerous efforts have been carried out in the last decades to
483 evaluate the protection conferred by approved and novel vaccines against
484 inhalational brucellosis. As mentioned, *Brucella* can enter through the respiratory
485 mucosa from where it spreads systemically, so an ideal vaccine should be able to
486 elicit mucosal protective immune responses to eliminate or reduce the spread of the
487 bacteria, but it should also generate a systemic protective response to prevent
488 infection of peripheral organs.

489 Currently, the vaccines in use for livestock are based on live attenuated strains
490 that prevent disease caused by *B. melitensis* (strain Rev.1) and *B. abortus* (strains
491 S19, RB51). Smither et al. demonstrated that subcutaneous (s.c.) administration of
492 the strain Rev.1 reduces the bacterial burden in the spleen but not in the lung or liver
493 of mice challenged with aerosolized *B. melitensis* 16M. However, i.n. immunization
494 with Rev.1 strain significantly reduces the burden of *B. melitensis* in the lung and
495 spleen at all times tested [30]. *B. abortus* strain RB51 is a rough strain approved to
496 prevent cattle brucellosis in the USA and other countries, and is preferably
497 administered by the s.c. route. Olsen et al. have shown that i.p. immunization with
498 RB51 strain does not protect from aerosol challenge with virulent *B. abortus* in mice
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499 [29]. In other study, administration of RB51 or the same strain over-expressing
500 superoxide dismutase by different routes (i.n., i.p., intradermal, s.c.) and prime-boost
501 strategies did not protect against i.n. *B. abortus* infection in mice [62]. However, i.n.
502 administration of RB51 together with TLR agonists (TLR2 or TLR4) significantly
503 increased protection in the lung [63], demonstrating that potentiating the immunity
504 with adjuvants, like TLR agonists, may be a useful strategy to improve the
505 performance of attenuated vaccines against respiratory infection. *B. abortus* strain
506 S19 is a smooth strain approved to prevent cattle brucellosis and is frequently
507 administered by the s.c. route. It has been shown that i.n. administration of S19
508 reduces the load of pathogenic *B. abortus* in the lung but does not modify the burden
509 of bacteria in the spleen [62]. These results demonstrate that the approved
510 attenuated vaccines against *B. abortus*, which have been shown to elicit protection
511 against parenteral challenge in mice models, do not protect efficiently against
512 respiratory challenge, which would explain at least in part their limited efficiency in
513 the protection of livestock.

514 Other studies have evaluated the protection against respiratory infection
515 conferred by experimental vaccines based on mutant strains of *Brucella*. In the
516 murine model, oral administration of *B. melitensis* WR201 managed to reduce the
517 bacterial load in lung and liver after intranasal challenge with *B. melitensis* 16M [64].
518 Similarly, i.n. vaccination of mice with high doses of *B. melitensis* Δ*znuA* (10^9
519 CFU/mice) conferred strong pulmonary protection against the i.n. challenge with *B.*
520 *melitensis* 16M and reduced its systemic dissemination [65]. Kahl-McDonagh et al.
521 demonstrated that *B. abortus* Δ*asp24* and *B. melitensis* Δ*asp24*, administered by i.p.
522 route, protect mice against homologous and heterologous aerosol challenge infection
523 [31]. However, the reduction in the burden of virulent *B. abortus* in the lung, although

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524 significant, was not as marked as that observed in the spleen, or in the organs of
525 animals challenged with *B. melitensis*. These results and those described above
526 show that the ability of these experimental vaccines to protect the lung from *Brucella*
527 infection may differ depending on the infecting *Brucella* species. In unvaccinated
528 mice the pulmonary load of aerosolized *B. melitensis* decreases more rapidly
529 compared to *B. abortus* [31]. This different behavior may also impact on the efficacy
530 of vaccination-induced clearance of lung bacteria.

531 While immunization with either approved or experimental vaccines based on
532 live mutant strains of *Brucella* may confer protection against respiratory challenge
533 with pathogenic brucellae, the use of such vaccines is associated with several safety
534 concerns that limit their use in animals and preclude their use in humans. Besides
535 their potential for reversion to a wild type phenotype, many of these strains still
536 produce clinical manifestations in humans and in some animals (e.g., abortion in
537 pregnant females). Moreover, the strains *B. abortus* RB51 and *B. melitensis* Rev.1,
538 currently approved for use in animal vaccination, are resistant to antibiotics
539 commonly used to treat human brucellosis. In the search for an efficient and safe
540 vaccine capable of protecting against respiratory challenge by *Brucella*, the
541 effectiveness of inactivated vaccines and subunit vaccines has been studied. Oral
542 immunization with different doses of gamma-irradiated *B. neotomae* showed that a
543 high dose (10^{11} CFU) is required to provide protection against i.n. *B. abortus*
544 challenge [66]. On the other hand, i.n. immunization of mice with *B. melitensis* LPS
545 together with outer membrane proteins of *N. meningitidis* group B as adjuvant,
546 induced a strong systemic and mucosal immune response that could control the
547 spread of *Brucella* to spleen and liver after respiratory infection, but was unable to
548 control infection at the lung level [67].

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549 A study that evaluated the immunogenicity and protection conferred by nasal
550 administration of Omp31 peptides in mice demonstrated a reduction in lung load
551 following the i.n. challenge with *B. melitensis*. Despite these promising results,
552 vaccination failed to control systemic dissemination [68]. In a recent study performed
553 by our group, i.n. administration of the *B. suis* BtaF adhesin in mice conferred high
554 levels of protection against intragastric *B. suis* infection. Unlike what was observed
555 for oral infection, nasal vaccination with BtaF did not protect against *B. suis*
556 respiratory infection [33]. In another recent study, i.n. immunization of mice with a
557 chitosan-based vaccine formulated with well-known *Brucella* antigens (SodC,
558 Omp19, BLS and PrpA) with or without *Brucella* LPS generated a humoral and
559 cellular immune response that reduced the burden of *B. abortus* 544 in lungs and
560 spleen after nasal challenge [69]. All these findings make it clear that protective
561 immune responses against *Brucella* spp. inhalational infection are intimately related
562 to the nature and composition of vaccines, the immunization route, and the *Brucella*
563 species used for challenge.
564

565 Currently, little is known about the immune response needed to achieve lung
566 protection during respiratory *Brucella* infection. Some studies concluded that CD8+ T
567 cells are critical for the resolution of infection, whereas others suggested that they
568 are dispensable [65,70]. Clapp et al. demonstrated that CD8+ T cells, but not CD4+
569 cells or IL-17, are essential for protection against respiratory infection [65]. In contrast
570 with this study, Yingst et al. demonstrated that CD8 KO mice are protected from
571 nasal challenge by oral vaccination with a live attenuated strain of *B. melitensis* [70].
572 As mentioned, in the murine model pulmonary protection against aerosolized *B.*
573 *melitensis* is conferred by IFN-ɣ-producing CD4+ T cells [60]. However, in the

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574 absence of this cellular population, CD8+ T cells can exert the protective response in
575 the lung. This compensatory mechanism between both cell populations could explain
576 the discrepancies in lung protection studies against inhalational brucellosis.

577 Recently, Wang et al. demonstrated that vaccination of mice with a strategy of
578 oral prime and nasal boost with high doses of a double-mutant of *B. abortus* called
579 znBAZ (which lacks *znuA* and *norD*) confers efficient protection against nasal
580 infection with virulent *B. a*bortus 2308, and its protective efficacy is superior to that of
581 the RB51 vaccine [71]. CD8+ T cells were essential for znBAZ-mediated protection
582 against the nasal challenge. In contrast, CD4+ T cells were required for protection
583 conferred by RB51. The znBAZ vaccine induces IFN-γ and TNF-α positive tissue-
584 resident memory CD8+ T cells (CD8+ T_{RM}), as well as polyfunctional cells in the lung.
585 CD8+ T_{RM} cells able to produce IL-17 were also induced by vaccination with znBAZ,
586 but neutralization of IL-17 in vivo did not affect protection. Vaccination with RB51
587 failed to induce CD4+ and CD8+ T_{RM} cells in the lung, which may explain its limited
588 ability to protect against respiratory infection by *B. abortus.* These results
589 demonstrate that the generation of T_{RM} cells is an important aspect to consider in the
590 development of new mucosal vaccines for respiratory *Brucella* infection.

591 In summary, lung protection studies demonstrate the difficulty of obtaining a
592 vaccine capable of generating protective responses against inhalational *Brucella*
593 infection. A possible explanation for this problem may be the inability of the tested
594 vaccines to generate an efficient innate and adaptive immune response in the
595 context of the lung mucosal microenvironment. Another possible explanation in the
596 case of live *B. abortus* vaccines is the ability of *Brucella* to suppress the innate
597 immune response in the lung as described by Hielpos et al. [28], which could affect
598 the ability of DCs to induce protective cellular immune responses. The intracellular

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599 nature of *Brucella* can also contribute to the inability of vaccines to induce efficient
600 protection. Once inhaled, *B. abortus* can infect and replicate in AM and pulmonary
601 epithelial cells without inducing potent activation of innate immunity [44,46,47,51].
602 This could allow the bacteria to avoid clearance or detection by adaptive immunity
603 effector mechanisms in a tolerogenic mucous environment. The studies reviewed
604 here suggest the need for further research to develop an efficient vaccine for
605 inhalational brucellosis.
606

607 **6. Concluding remarks**

608 Understanding the pathogenesis and immune response to inhalational *Brucella*
609 infection is an important issue given the prevalence of brucellosis and the frequency
610 of infection by the respiratory route in humans and animals. Data from human cases
611 and animal models have clearly shown that *Brucella* can rapidly disseminate from its
612 pulmonary site of entry to peripheral organs. In the lung, however, the inflammatory
613 reaction is scarce. Pieces of evidence collected from these studies help to
614 understand the reasons for the efficiency of the respiratory route for *Brucella*
615 infection. On the one hand, at least some *Brucella* species seem to establish
616 persistent infections in lung tissues. This may be related to the ability of *Brucella* to
617 survive and replicate in lung epithelial cells and AM, its capacity to modulate the
618 pulmonary inflammatory response, its resistance to locally produced antimicrobial
619 peptides, and its ability to downmodulate MHC-II expression and antigen
620 presentation by AM. On the other hand, several studies have shown that *Brucella*
621 can rapidly reach the bloodstream and peripheral organs from its initial site of entry in
622 the lungs. This dissemination seems to be executed, at least in part, by infected AM
623 that act as Trojan horses, and happens even before an enhanced innate immune

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624 response can be mounted in the lungs. Therefore, although pulmonary innate
625 receptors (TLRs, inflammasomes) and cytokine responses have been shown to exert
626 some control of *Brucella* infection, these factors are insufficient to avoid the systemic
627 dissemination of the pathogen from the lungs, at least during the early phase of lung
628 infection. Therefore, the challenge is to develop human vaccines that could ideally
629 control pulmonary *Brucella* infection and could also prevent systemic spread. The
630 studies performed with live attenuated strains in animal models have shown the
631 difficulty to protect efficiently against respiratory challenge. Nevertheless, it has been
632 shown that IL-17 is involved in the early control of the pulmonary infection, and IFN-γ
633 is crucial for late control in all organs after respiratory challenge. In addition, both
634 CD4+ and CD8+ cells seem to mediate these responses. Therefore, it can be
635 presumed that lung colonization and systemic spread of *Brucella* after respiratory
636 infection could be prevented by immunization protocols eliciting these types of
637 responses.
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640
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647
648 **Conflict of interest**

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649 The authors declare no conflict of interest.

650

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861 **Figure legends**

862

863 **Figure 1**. **Interactions of *Brucella* spp. with lung cells**. The scheme summarizes
864 the results obtained from in vivo studies in mice and in vitro studies performed with
865 human and mouse cells, using different *Brucella* species. Upon inhalation *Brucella*
866 would interact with alveolar and bronchial epithelial cells and alveolar macrophages
867 (AM) eliciting the secretion of cytokines and chemokines (solid red lines). In turn,
868 some of these soluble factors would stimulate the production of chemokines and
869 defensins (hBD2) by adjacent cells (dashed lines). Some alveolar macrophages
870 containing viable *Brucella* can migrate to the mediastinal lymph nodes (MdLN),
871 presumably contributing to the systemic dissemination of the pathogen. In the lymph
872 node, CD4+ and CD8+ naïve T cells are stimulated by antigen presenting cells
873 (APC), the identity of which remains to be established. Th1, Th17 and CD8+ cells
874 have been shown to contribute to *Brucella* control in the lung. *Brucella* opposes
875 several evasion mechanisms to these immune responses (blue lines) including the
876 downmodulation of TLR signaling, the resistance to beta-defensins and the
877 downmodulation of MHC-II expression in alveolar macrophages. ATI: type I alveolar
878 epithelial cells, ATII: type II alveolar epithelial cells.

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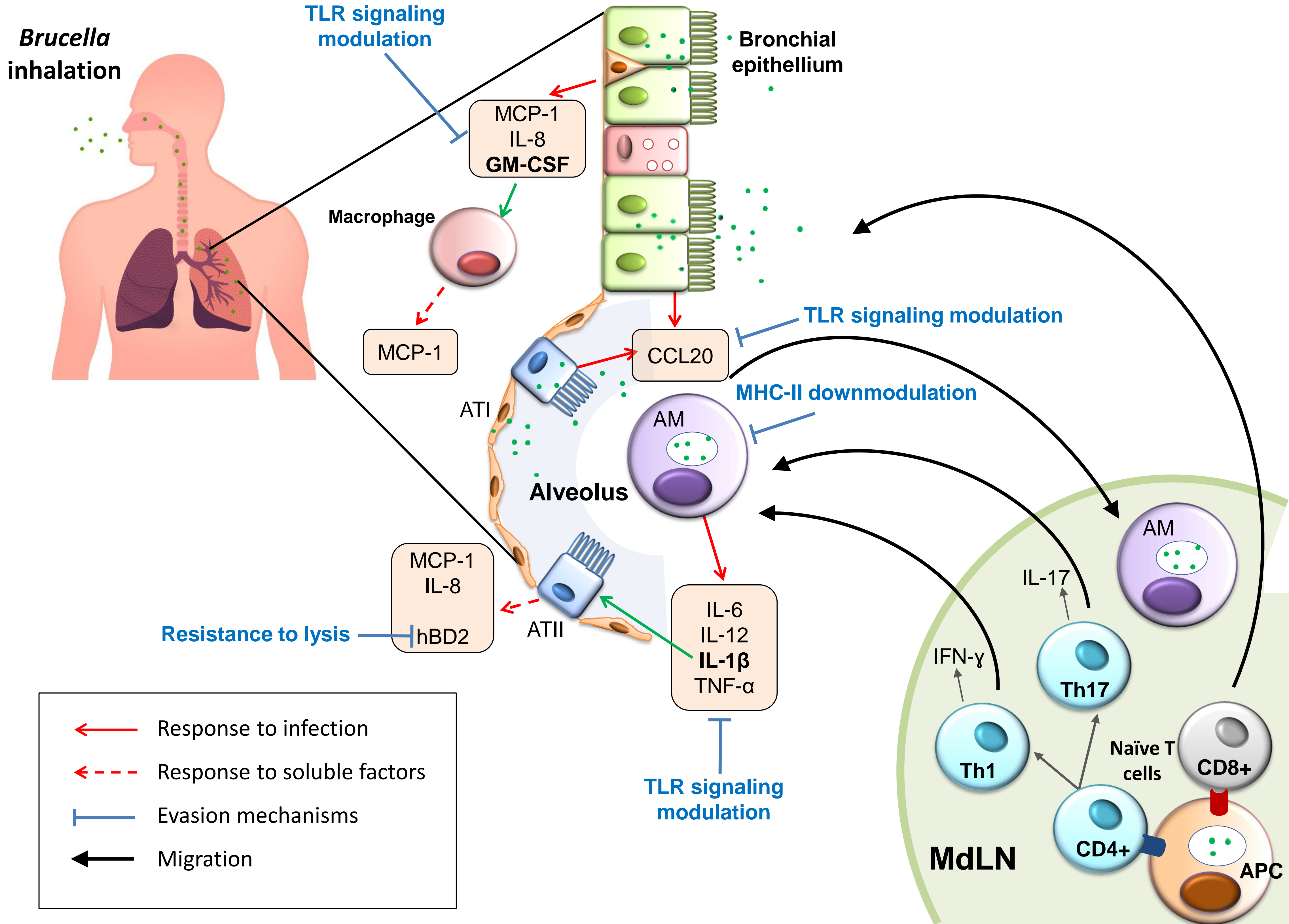

Brucella inhalation
TLR signaling modulation
Bronchial epithellium
MCP-1
IL-8
GM-CSF
Macrophage
MCP-1
CCL20
TLR signaling modulation
ATI
MHC-II downmodulation
AM
Alveolus
AM
MCP-1
IL-8
hBD2
Resistance to lysis
ATII
IL-6
IL-12
IL-1β
TNF-α
IL-17
IFN-γ
Th17
Th1
Naïve T cells
CD8+
CD4+
APC
MdLN
TLR signaling modulation
Response to infection
Response to soluble factors
Evasion mechanisms
Migration