Lack of Interleukin-6 Affects IFN-γ and TNF-α Production and Early In Vivo Control of *Brucella abortus* Infection

Erika S. Guimarães 1,2, Jéssica M. Martins 2, Marco Túlio R. Gomes 2, Daiane M. Cerqueira 2,* and Sergio C. Oliveira 2,*

1 Department of Genetics, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte 31270-901, Brazil; erikasousaguimaraes@gmail.com
2 Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte 31270-901, Brazil; jezaid@hotmail.com (J.M.M.); mtrgomes19@gmail.com (M.T.R.G.); daianecerqueira@yahoo.com.br (D.M.C.)
* Correspondence: scozeus1@gmail.com

Received: 12 November 2020; Accepted: 9 December 2020; Published: 11 December 2020

**Abstract:** Interleukin-6 (IL-6) is a pleiotropic cytokine promptly produced in response to infections, which contributes to host defense through the stimulation of acute phase immune responses. *Brucella abortus* is an intracellular bacterium that causes chronic disease in humans and domestic animals and triggers a robust immune response, characterized by the production of inflammatory cytokines. However, the mechanisms of IL-6-related immune responses in the context of *Brucella* infections are not completely understood. In this report, we describe an increased susceptibility of IL-6 knockout (KO) mice in the early phase of *Brucella* infection. Furthermore, we demonstrate that IL-6 is required for interferon (IFN)-γ and tumor necrosis factor (TNF)-α induction by infected splenocytes, indicating a protective role for IL-6 against *B. abortus* that parallels with Th1 type of immune response. Additionally, IL-6 KO mice exhibited reduced splenomegaly during the early phase of the infection. Corroborating this result, IL-6 KO mice displayed reduced numbers of macrophages, dendritic cells, and neutrophils in the spleen and reduced myeloperoxidase activity in the liver compared to wild-type infected mice. However, we demonstrate that IL-6 is not involved in *B. abortus* intracellular restriction in mouse macrophages. Taken together, our findings demonstrate that IL-6 contributes to host resistance during the early phase of *B. abortus* infection in vivo, and suggest that its protective role maybe partially mediated by proinflammatory immune responses and immune cell recruitment.

**Keywords:** IL-6; *Brucella abortus*; innate immunity; neutrophils; cell recruitment

1. **Introduction**

Interleukin-6 (IL-6) is an immunoregulatory cytokine with a broad effect on cells of the immune system [1], and high levels of IL-6 are detected during tissue damage, microbial infection, and several other inflammatory conditions [2,3]. Gram-negative bacteria induce increased plasma levels of IL-6 during inflammation, and IL-6 expression has been proposed as a critical activator of protective immunity [4–6]. Several studies have shown that IL-6 acts as a powerful inflammatory cytokine that is essential for immune responses in distinct scenarios [7–9]. For instance, IL-6 is crucial for inducing an acute-phase inflammatory response against *Listeria monocytogenes* [10] and for the production of interferon-gamma (IFN-γ) during *Chlamydia trachomatis* [11] and *Mycobacterium tuberculosis* infections [6,12]. Furthermore, IL-6 is also required for the production of tumor necrosis factor (TNF-α) during *M. tuberculosis* infection [6].
Brucella abortus is a Gram-negative, facultative intracellular coccobacillus, the causative agent of brucellosis, a chronic inflammatory zoonotic disease in humans and livestock. In humans, the disease symptoms include undulant fever and endocarditis arthritis. In animals, it leads to abortion and infertility, resulting in serious economic losses [13,14]. In mice, infection with B. abortus provokes rapid activation of the innate immune system, which includes phagocytosis by professional phagocytes, such as macrophages, dendritic cells, and neutrophils. The recognition of bacteria by pattern recognition receptors and cytokine secretion are essential for the restriction of bacterial replication [15]. In particular, the production of Th1-type proinflammatory cytokines, such as IFN-γ and tumor necrosis factor TNF-α, is crucial for the early control of B. abortus [16–18].

Recruitment of immune cells to the site of infection is a hallmark for many systemic bacterial infections. Previous data showed that IL-6 is closely linked to immune cell influx, and IL-6 deficient mice are unable to induce neutrophilia in the bloodstream following bacterial challenge [19,20]. Furthermore, we have recently shown that neutrophils are crucial for the control of bacteria replication, whereas depletion of these cells renders mice more susceptible to Brucella infection [21]. However, the implications of IL-6 in the context of Brucella infection are not completely understood, especially due to the lack of studies using IL-6 knockout (KO) mice.

In the present study, we have used IL-6 KO mice to evaluate the role of this cytokine during B. abortus infection and provide novel insights on the relationship between IL-6 and innate immune responses in vivo, including the production of other inflammatory cytokines and immune cell recruitment in the context of B. abortus infection. Our results indicate that IL-6 plays a protective role in the immune response against B. abortus and is crucial for inducing a full inflammatory response. Additionally, we established the importance of IL-6 for inflammatory cell recruitment to the site of the infection, since IL-6 KO infected mice showed a reduced number of macrophages, dendritic cells, and neutrophils in spleens compared to wild-type infected mice. Taken together, these data show the beneficial aspect of IL-6 production and reveal an important role of this cytokine during B. abortus infection and pathogenesis.

2. Results

2.1. IL-6 Is Not Required for In Vitro Control of Brucella Infection

The cytokine IL-6 is largely described for its protective role in immune responses against bacterial infections [10,19,20]. Therefore, to evaluate whether IL-6 affects Brucella intracellular replication, we infected wild-type (C57BL/6) and IL-6 KO macrophages with a 100:1 multiplicity of infection (MOI) and quantified the number of B. abortus colony forming unit (CFU) in vitro 3 h after infection until 72 h post-infection.

Wild-type and IL-6 KO infected macrophages showed similar numbers of B. abortus at all time intervals analyzed (Figure 1A). To further explore this data, and to evaluate if this result was associated with Brucella intracellular infection levels, we performed experiments using a lower MOI (10:1). Nevertheless, lower infection levels also resulted in no difference in CFU numbers between the wild-type and IL-6 KO macrophages at all time intervals studied (Figure 1B), indicating that this event is not associated with the initial Brucella numbers. In accordance with these data, confocal images showed similar levels of intracellular B. abortus–GFP in wild-type and IL-6 KO macrophages 24 h post-infection (Figure 1C). These findings indicate that IL-6 is not involved in the control of B. abortus replication in macrophages.
Figure 1. Interleukin (IL)-6 is not involved in *B. abortus* intracellular growth in macrophages. Macrophages derived from wild-type (C57BL/6) and IL-6 KO mice were infected with *B. abortus* for 3, 24, 48, or 72 h. (A) Residual CFU in macrophages derived from wild-type and IL-6 KO mice infected with *B. abortus* with MOI = 100:1. (B) Residual CFU in macrophages derived from wild-type and IL-6 KO mice infected with *B. abortus* MOI 10:1. (C) Macrophages derived from wild-type (C57BL/6) and IL-6 KO mice were infected with *B. abortus*–GFP (MOI = 100:1). Fluorescence microscopy analysis of *B. abortus*–GFP replication 24 h post-infection. Images are representative of all experiments analyzed. GFP-expressing bacteria are shown in green (*Brucella*–GFP) and 4′,6-diamidino-2-phenylindole (DAPI) (DNA) is in blue. Scale bar = 30 mm. Data are representative of three independent experiments.

2.2. TNF-α Production by Macrophages Infected with *B. abortus* Is Partially Dependent on IL-6

The protective role of IL-6 during bacterial infections is regularly mediated through the regulation of other inflammatory cytokines [10,22]. Hence, in order to investigate the role of IL-6 in cytokine production during *B. abortus* infection, macrophages of wild-type and IL-6 KO mice were infected with different *B. abortus* MOIs for 24 h, and cytokine secretion evaluated. Our results show that *B. abortus* infection induced IL-6 secretion in all analyzed conditions (Figure 2A). Furthermore, as expected, the absence of IL-6 (IL-6 KO mice) resulted in abolished IL-6 secretion (Figure 2A) during *B. abortus* infection. As a control, macrophages were treated with lipopolysaccharides (LPS) and, as expected, LPS induced IL-6 secretion in wild-type mice, but not in IL-6 KO (Figure 2A).
In order to investigate IL-6 participation in regulating other immune responses, we evaluated the secretion of the proinflammatory cytokines IL-12 and TNF-α and anti-inflammatory cytokine IL-10 in wild-type and IL-6 KO infected macrophages. IL-6 KO infected macrophages showed similar levels of IL-12 secretion (Figure 2B) in all infection conditions analyzed, compared to wild-type infected macrophages. Additionally, IL-6 KO macrophages stimulated with LPS showed similar IL-12 secretion levels compared to wild-type cells (Figure 2B). Moreover, in the absence of IL-6, IL-10 secretion was slightly enhanced only upon infection with lower bacterial loads (Figure 2C) while IL-12 is not a secreted cytokine in all infection conditions. Furthermore, IL-6 KO macrophages stimulated with LPS showed similar IL-12 secretion (Figure 2B) in all infection conditions analyzed, compared to wild-type infected macrophages.

Taken together, these results show that TNF-α production is reduced in macrophages in the absence of IL-6, while IL-10 is mildly altered only with lower bacterial loads, and IL-12 is not affected by IL-6, indicating that IL-6 has a role in regulating TNF-α secretion during B. abortus infection.

2.3. IL-6 Is Required for Full Resistance During the Early Phase of B. abortus Infection In Vivo

Our results demonstrated that IL-6 KO macrophages showed reduced TNF-α secretion (Figure 2D). Furthermore, impaired IL-6 function causes enhanced susceptibility of mice to infection by various bacterial pathogens [4,11,23]. Therefore, in order to evaluate the role of IL-6 in host resistance against Brucella infection in vivo, we infected wild-type and IL-6 KO mice with B. abortus, and CFU counts were treated with 1 µg/mL of E. coli LPS for 24 h. The concentration of (A) IL-6, (B) IL-12, (C) IL-10, and (D) tumor necrosis factor (TNF)-α by Enzyme-Linked Immunosorbent Assay (ELISA) in the culture supernatants was estimated by ELISA. Data are representative of three independent experiments. * p < 0.05 compared to wild-type infected with B. abortus, via one-way ANOVA.

Figure 2. IL-6 modulates proinflammatory cytokine secretion during B. abortus infection. Macrophages derived from wild-type (C57BL/6) and IL-6 KO mice were left non-infected (NI) or infected with B. abortus (Ba) with MOIs of 1:10, 5:10, or 100:1, as indicated, for 24 h. As a positive control, macrophages were treated with 1 µg/mL of E. coli LPS for 24 h. The concentration of (A) IL-6, (B) IL-12, (C) IL-10, and (D) tumor necrosis factor (TNF)-α by Enzyme-Linked Immunosorbent Assay (ELISA) in the culture supernatants was estimated by ELISA. Data are representative of three independent experiments. * p < 0.05 compared to wild-type infected with B. abortus, via one-way ANOVA.
were analyzed at 1 and 6 weeks post-infection in spleens, an organ that serves as a niche for this bacterium. IL-6 KO mice showed enhanced bacterial numbers at 1 week post-infection compared to wild-type mice, whereas similar bacterial numbers were assessed at 6 weeks post-infection compared to infected wild-type mice (Figure 3A).

![Figure 3.](image)

**Figure 3.** Suppression of IL-6 enhances susceptibility to *B. abortus* infection in mice. Wild-type (C56BL/6) and IL-6 KO mice were infected intraperitoneally with $1 \times 10^6$ CFU of *B. abortus*. Mice were sacrificed at 1 week and 6 weeks post-infection, spleens were weighed, and diluted spleen homogenates were added to Brucella Broth (BB) medium agar plates for CFU determination. (A) Residual *B. abortus* CFU in the spleen of wild-type and IL-6 KO mice were determined at 1 week (wk) and 6 weeks post-infection. (B) Spleen weight (mg) of wild-type and IL-6 KO infected mice were determined at 1 week and 6 weeks post-infection. Data are representative of three independent experiments. Data are mean ± SD of five mice/group. *p < 0.05 compared to wild-type infected with *B. abortus*, via two-way ANOVA.

Additionally, we evaluated the weight of the spleens of wild-type and IL-6 KO infected mice. Our results demonstrated that IL-6 KO mice showed reduced spleen weight at 1 week post-infection (Figure 3B), and similar spleen weight at 6 weeks post-infection compared to wild-type infected mice (Figure 3B).

Taken together, these results demonstrate that IL-6 is required for early host resistance against *Brucella* infection in vivo, and indicate that IL-6 is required for the splenomegaly induced by *B. abortus*.

### 2.4. Lack of IL-6 Leads to Reduced Type 1 Immune Response to *B. abortus*

IL-6 is considered an activator of acute phase inflammatory responses [23,24]. Moreover, the production of inflammatory cytokines, such as IFN-γ and TNF-α, is associated with the control of *B. abortus* infection [25–27]. Here, we have showed that IL-6 KO mice were more susceptible to *Brucella* infection than wild-type animals during the acute phase of the infection (Figure 3A). Therefore, in order to further analyze the relevance of IL-6 in vivo, we investigated whether the increased susceptibility of IL-6 KO mice during *B. abortus* infection was correlated with altered patterns of cytokine secretion in mice. Splenocytes from wild-type and IL-6 KO mice infected with *B. abortus* for 1 week and 6 weeks were stimulated with live bacteria, or the controls ConA or LPS, and cytokine secretion was measured.

IL-6 KO infected mice produced lower levels of the proinflammatory cytokines IFN-γ (Figure 4A,C) and TNF-α (Figure 4B,D), at 1 week and 6 weeks post-infection compared to wild-type infected animals. Furthermore, stimulation with the controls ConA or LPS, respectively, resulted in similar IFN-γ and TNF-α levels, demonstrating the specificity of the response.
we showed that IL-6 KO mice are more susceptible to early Brucella infection. Additionally, IL-6 KO mice showed reduced splenomegaly at 1 week post-infection (Figure 3B).

To further evaluate the potential role of IL-6 in neutrophil recruitment, we submitted liver homogenates from wild-type and IL-6 KO mice infected with *B. abortus* to myeloperoxidase (MPO) assay to indirectly determine neutrophil recruitment. Wild-type infected animals displayed enhanced MPO activity in the livers compared to non-infected animals (Figure 5D), demonstrating that IL-6 KO infected mice showed reduced number of macrophages (Figure 5A), dendritic cells (Figure 5B), and neutrophils (Figure 5C) in the spleens compared to infected wild-type mice. To further investigate the recruitment of immune cell populations to the site of infection, we investigated the recruitment of inflammatory immune cells during infection and inflammation [9,24,29]. Herein, we showed that IL-6 KO mice are more susceptible to early *Brucella* infection in vivo (Figure 3A). Additionally, IL-6 KO mice showed reduced splenomegaly at 1 week post-infection (Figure 3B).

In order to investigate the possible mechanism involved in the increased susceptibility and reduced spleen weight in IL-6 KO mice, we investigated the recruitment of immune cell populations during *B. abortus* infection. Wild-type and IL6-KO mice were infected with *B. abortus*, and 1 week post-infection the number of macrophages, dendritic cells, and neutrophils was evaluated in the spleens by flow cytometry.

IL-6 KO infected mice showed reduced numbers of macrophages (Figure 5A), dendritic cells (Figure 5B), and neutrophils (Figure 5C) in the spleens compared to infected wild-type mice. To further investigate the role of IL-6 in immune cell recruitment, we evaluated the number of macrophages, dendritic cells, and neutrophils in the spleens of infected mice 1 week post-infection. The number of these cells was determined by flow cytometry and was found to be significantly reduced in IL-6 KO mice compared to wild-type infected mice. This suggests that IL-6 plays a crucial role in the recruitment of immune cells during *B. abortus* infection.

**Figure 4.** IL-6 is required for interferon (IFN)-γ and TNF-α production by infected splenocytes. Splenocytes of infected mice were stimulated in vitro with *B. abortus* (Ba), 5 µg/mL ConA, 1 µg/mL LPS, or medium (NI) as a negative control. Splenocyte supernatants from 1 week-infected mice were harvested 48 or 72 h after stimulation and measured by ELISA for (A) IFN-γ and (B) TNF-α. Splenocyte supernatants from 6 week-infected mice were harvested 48 or 72 h after stimulation and measured by ELISA for (C) IFN-γ and (D) TNF-α. Data are representative of three independent experiments. Data are mean ± SD of five mice/group. *p < 0.05 compared to wild-type infected with *B. abortus*, via two-way ANOVA.

Th1 cells are primarily defined by their ability to secret IFN-γ and TNF-α. Furthermore, IFN-γ is known to be the major Th1-inducing cytokine, leading to the polarization of a Th1 phenotype [28,29]. Therefore, these results indicate the ability of IL-6 to regulate, at least partially, immune responses that seem to parallel with type 1 immunity in vivo during *B. abortus* infection.

2.5. IL-6 Regulates Innate Immune Cell Recruitment

A typical response to bacterial infections is the immediate recruitment of inflammatory immune cells to the site of infection. Furthermore, it was broadly demonstrated that IL-6 is involved in the recruitment of inflammatory immune cells during infection and inflammation [9,24,29]. Herein, we showed that IL-6 KO mice are more susceptible to early *Brucella* infection in vivo (Figure 3A). Additionally, IL-6 KO mice showed reduced splenomegaly at 1 week post-infection (Figure 3B).
evaluate the potential role of IL-6 in neutrophil recruitment, we submitted liver homogenates from wild-type and IL-6 KO mice infected with *B. abortus* to myeloperoxidase (MPO) assay to indirectly determine neutrophil recruitment. Wild-type infected animals displayed enhanced MPO activity in the livers compared to non-infected animals (Figure 5D), demonstrating that *B. abortus* infection induces neutrophil recruitment. Furthermore, corroborating the reduced neutrophil recruitment in IL-6 KO infected mice spleens compared to wild-type infected mice (Figure 5C), IL-6 KO mice displayed diminished MPO activity (Figure 5D) compared to liver homogenates from wild-type infected mice. Taken together, these findings indicated that IL-6 plays a crucial role in the recruitment of immune cells during *B. abortus* infection and is required for the *Brucella*-induced recruitment of neutrophils.

![Figure 5](image-url)

**Figure 5.** IL-6 regulates immune cell recruitment. Spleen cells from 1 week-infected wild-type (C57BL/6) and IL-6 KO mice were stained ex vivo for flow cytometry analysis. Cells were assessed for (A) macrophages (Cd11b+F4/80+), (B) dendritic cells (Cd11b+Cd11c+), and (C) neutrophils (Cd11b+Ly6G+). Data represent the number of labeled cells (Cd11b+F4/80+, Cd11b+Cd11c+, or Cd11b+Ly6G+) per number of spleen cells. (D) Liver homogenates from wild-type and IL-6 KO non-infected (NI) mice or those infected with *B. abortus* (Ba) were submitted to a myeloperoxidase (MPO) assay. Data are mean ± SD of five mice/group. Data are representative of two independent experiments. * p < 0.05 compared to wild-type (C57BL/6) non-infected (NI) mice; # p < 0.05 compared to wild-type infected mice (A–C) via Student’s *t*-test, (D) via two-way ANOVA.

### 3. Discussion

IL-6 has emerged as a key cytokine during bacterial infections, with complex effects on cells of the immune system and context-dependent proinflammatory described properties [1,30]. Moreover, IL-6 deficiency leads to impaired innate and adaptive immunity to parasitic, viral, and bacterial infections [29,31,32]. Corroborating previous studies [26,33,34], we showed here that *Brucella* induces IL-6 secretion, and it was recently shown that patients with brucellosis exhibit higher IL-6 levels compared to control patients [31]. However, the role of IL-6 during *Brucella* infection is still poorly understood.
In this study, we provided new insights on the relationship between IL-6 and B. abortus in vivo infection. We demonstrated that IL-6 is important for the control of B. abortus during the early phase of the infection, and demonstrated that the enhanced susceptibility in IL-6 KO mice occurs in conjunction with a decrease in type 1 cytokine secretion and reduced inflammatory cell recruitment.

Previous data demonstrated that macrophages treated with an anti-IL6 monoclonal antibody displayed enhanced susceptibility to B. abortus infection in macrophages, suggesting that the induction of IL-6 is required for bacterial killing [32]. Here, we showed that IL-6 is not involved in the control of bacterial replication in macrophages within all analyzed times and infection conditions, indicating that IL-6 is not required for the control of B. abortus replication in IL-6 KO macrophages. The discrepancy between these results might be associated with the use of anti-IL6 antibodies in the previous study, since independent studies have shown surprisingly enhanced levels of IL-6 in animals treated with anti-IL-6 antibodies [33–35]. In accordance with our data, it was previously reported that IL-6 plays no role in the intracellular growth of B. abortus in macrophages [25].

Furthermore, we demonstrated here that IL-6 is critical for the induction of TNF-α secretion in macrophages infected with B. abortus, corroborating previous data showing that macrophages treated with IL-6 small interfering RNA (siRNA) and infected with B. abortus show reduced TNF-α secretion [32].

Corroborating previous reports demonstrating that IL-6 is often correlated with the susceptibility to infection in several bacterial models [10,19,20], we demonstrated that IL-6 is required for the control of Brucella replication in vivo during the early phase of the infection. Accordingly, IL-6 is traditionally considered an activator of acute-phase inflammatory responses [10,24,25].

We demonstrated that the increased bacterial numbers in the spleens of IL-6 KO mice was accompanied by decreased levels of IFN-γ and TNF-α, proinflammatory cytokines critically associated with the control of B. abortus infection [25,27]. Indeed, previous studies have demonstrated that IFN-γ KO mice succumb to brucellosis rapidly after infection [16]. Furthermore, IL-6-dependent reduction of IFN-γ was also described for other bacterial infections [6,36]. Additionally, a blockade of TNF-α using specific antibodies likewise increases susceptibility to B. abortus [18,37]. Therefore, our findings provide evidence that IL-6 has a protective role in the control of Brucella in vivo that is possibly associated with the production of type 1 proinflammatory cytokines. These data support previous findings indicating that IL-6 promotes a Th1 phenotype in inflammatory diseases [36,38–40], and also corroborates findings showing that mice treated with an anti-IL-6 antibody and infected with B. abortus show reduced IFN-γ, TNF-α, and IL-12 secretion [32].

Here, we demonstrate that despite enhanced bacterial numbers during the early phase of the infection, IL-6 KO mice showed reduced spleen weight at 1 week post-infection. This reduced splenomegaly in IL-6 KO infected mice is most likely related to the impaired recruitment of macrophages, dendritic cells, and neutrophils to the spleens in these mice. Indeed, it was previously shown that injection of recombinant IL-6 into wild-type mice causes an increase in neutrophilia [41]. Furthermore, this result confirms extensive previous studies showing that IL-6 modulates the accumulation of neutrophils during bacterial infections and inflammation [8,19,20,41].

Furthermore, it is well-known that the recruitment of leukocytes is dependent on the specificity of chemokines produced in the inflammatory site. In this regard, interleukin-8 (IL-8) is the most important chemokine for the recruitment of polymorphonuclear cells [42]. It was previously shown that the IL-6-dependent leukocyte recruitment in vivo is linked to the fact that the IL-6–soluble interleukin 6 receptor alpha (sIL-6Ra) complex can activate endothelial cells to secrete IL-8 [43]. Therefore, the IL-6 role in regulating neutrophils recruitment during B. abortus infection could be related to the production of other cytokines and chemoattractants in an IL-6-dependent pathway. Additionally, apoptosis has been implicated in the clearance of neutrophils from sites of inflammation [44], and IL-6 was shown to delay neutrophil apoptosis [45]. Therefore, another possible explanation for the reduced neutrophil numbers in IL-6 KO mice is that IL-6 could affect neutrophil apoptosis and removal from the circulation,
enabling neutrophil survival during B. abortus infection. However, the mechanisms linking IL-6 and neutrophils recruitment during B. abortus infection remain to be elucidated.

Additionally, it was recently demonstrated that neutrophils are crucial for the control of Brucella replication during the early phase of the infection, since depletion of neutrophils renders wild-type mice more susceptible to Brucella infection [21], demonstrating the importance of neutrophils in controlling the early stages of B. abortus infection.

In conclusion, our findings revealed that IL-6 enhances type 1 cytokine responses and the recruitment of immune cells during the early stage of infection, two events that are critically associated with the control of Brucella infection. Taken together, these data show IL-6 acting as an important regulator of immunity during brucellosis, and imply that IL-6 is a promising therapeutic target during bacterial infections.

4. Materials and Methods

4.1. Mice

Wild-type C57BL/6 mice were purchased from the Federal University of Minas Gerais, and IL-6 knockout (KO) mice were generated in the C57BL/6 background. Genetically deficient and control mice were maintained at our facilities at the Federal University of Minas Gerais and used at 6–8 weeks of age. Mice were housed in filter-top cages and provided with sterile water and food ad libitum. All animal experiments were preapproved by the Institutional Animal Care and Use Committee of the Federal University of Minas Gerais (CEUA no. 165/2019).

4.2. Bacterial Strains

Bacteria used in this study included the B. abortus virulent strain S2308 and a variant that constitutively expresses GFP, both obtained from our laboratory collection. Before being used for cell infection, bacteria were grown in Brucella broth medium (BD Pharmingen, San Diego, CA, USA) for 3 days at 37 °C under constant agitation.

4.3. Cell Culture

Macrophages were derived from the bone marrow of C57BL/6 and IL-6 KO mice and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) medium, as previously described [46]. Briefly, bone marrow cells were removed from the femurs and tibias of the animals and cultured in 24-well plates (5 × 10^5 cells per well for cytokine analysis, and 1 × 10^5 cells per well over a sterile coverslip for microscopy analysis) in DMEM (Life Technologies) containing 10% fetal bovine serum (FBS) (Life Technologies), 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (Life Technologies), 100 U/mL penicillin (Life Technologies), and 10% L929 cell-conditioned medium, as the source of Macrophage Colony Stimulating Factor (M-CSF) for macrophages at 37 °C in 5% CO₂.

4.4. Infection with B. abortus

Cultured cells were infected in vitro with virulent B. abortus strain 2308 with the indicated MOIs (see figure legends) in DMEM (Life Technologies), supplemented with 10% FBS (Life Technologies), for 24 h. In confocal microscopy experiments, macrophages were infected with B. abortus–GFP at an MOI of 100:1 DMEM supplemented with 10% FBS (Life Technologies) for 24 h.

4.5. Measurement of Brucella CFU in Macrophages

For the measurement of viable intracellular bacteria using CFU, cells infected with the described MOI were washed twice 3 h, 24 h, 48 h, and 72 h post-infection with PBS (Life Technologies), and then lysed for 10 min at room temperature in 1 mL of PBS containing 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) under manual agitation. Lysates were diluted from 10 to 1000 times in PBS and
plated on petri dishes containing *Brucella* broth agar. Petri dishes were incubated for 3 days at 37 °C before CFU counting.

4.6. Estimation of Intracellular *Brucella* Numbers by Confocal Microscopy

Macrophages were infected as described above with *Brucella*-GFP (MOI of 100:1) and the bacteria number was assessed in cells infected for 24 h, as previously described [47]. Cells were washed twice with PBS and fixed in 4% paraformaldehyde (pH 7.4) at room temperature for 30 min. After fixation, coverslips were washed three times with PBS. Coverslips were mounted in slides using ProLong Gold with DAPI mounting medium (Invitrogen, Carlsbad, California, United States). Confocal microscopy analysis was performed in a Nikon A1 confocal system. Three coverslips were analyzed per sample and images were taken using a 10× objective for six random areas of each coverslip. Images presented here are representative.

4.7. Bacterial Enumeration in *B. abortus*-Infected Mice

Five mice per group (C57BL/6 and IL-6 KO) were infected intraperitoneally (i.p.) with $1 \times 10^6$ virulent *B. abortus* in 100 mL of PBS. After 1 week and 6 weeks post-infection, animals were sacrificed, and spleens were weighed and used to determine the number of bacterial CFU, as previously described [48]. Organs harvested from each animal were macerated in 10 mL of saline (NaCl 0.9%). To determine total bacterial burden, spleens were serially diluted in saline and plated in duplicate on *Brucella* broth agar. Plates were incubated for 3 d at 37 °C, and CFU number was determined.

4.8. Spleen Cell Cultures and Flow Cytometry Analysis

Five mice from each group (C57BL/6 and IL-6 KO) were infected i.p. with $1 \times 10^6$ CFU *B. abortus* and sacrificed at 1 week post-infection. Spleen cells were harvested treated with an ammonium–chloride–potassium buffer (0.15 M NH$_4$Cl, 1.0 mM KHCO$_3$, 0.1 mM ethylenediaminetetraacetic acid (EDTA); pH 7.2) to lyse red blood cells. After washing, cells were adjusted to $1 \times 10^6$ cells per well in a 96-well plate in RPMI medium (Life Technologies) supplemented with 2 mM L-glutamine (Life Technologies), 25 mM HEPES, 10% heat-inactivated FBS, penicillin G sodium (100 U/mL), and streptomycin sulfate (100 mg/mL). The ex vivo staining of cells was performed as previously described [21]. Briefly, cells were centrifuged at 1500 rpm for 7 min at 4 °C and washed with PBS containing 1% bovine serum albumin (PBS/BSA). Cells were incubated with anti-CD16/CD32 (FcBlock) (1:30 diluted in PBS/BSA) for 20 min at 4 °C. The cells were then centrifuged and washed in PBS/BSA and incubated for 20 min at 4 °C with a mixture of the following antibodies: rat IgG2a anti-murine F4/80 conjugated to biotin (clone BM8; 1:200); rat IgG2b anti-murine CD11b conjugated to APC-Cy7 (clone M1/70; 1:200); hamster IgG1 anti-murine CD11c conjugated to FITC (clone HL3; 1:200); rat IgG2a anti-murine Ly-6G conjugated to PE (clone 1A8; 1:200) and rat IgG2a anti-murine CD62L conjugated to APC (clone MEL-14; 1:400). All antibodies were obtained from BD Pharmingen. Cells were centrifuged and washed again with PBS/BSA and incubated with streptavidin conjugated to PerCP Cy5.5 (1:30) for 20 min at 4 °C. Finally, cells were washed three times, suspended in PBS buffer, and evaluated using Attune Acoustic Focusing equipment (Life Technologies). Results were analyzed using FloWJo software (Tree Star, Ashland, OR, United States) and represent the number of labeled cells (macrophages: Cd11b$^+$ F4/80$^+$; dendritic cells: Cd11b$^+$ Cd11c$^+$; or neutrophils: Cd11b$^+$ Ly6G$^+$) per number of spleen cells.

4.9. Cytokine Measurements

For cytokine determination, macrophages were seeded at a density of $5 \times 10^5$ cells per well in 24-well plates and infected with the described *B. abortus* MOI, as described above, for 24 h. As a positive control, cells were primed with 1 μg/mL of *E. coli* LPS (Sigma) for 24 h. Then, supernatants from cell culture were harvested and assayed for the production of murine IL-6, IL-12, IL-10, and TNF-α by ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions. For the
in vivo experiments, spleen cells were harvested from five infected mice from each group (C57BL/6 and IL-6 KO) at 1 week and 6 weeks post-infection, as described above. To measure cytokine concentration by ELISA, splenocytes were stimulated with *B. abortus* at an MOI of 100:1, 1 µg/mL *Escherichia coli* LPS (Sigma), or 5 µg/mL concanavalin A (ConA) (Sigma). Unstimulated cells were used as the negative control (NI). Spleen cells were incubated at 37 °C in 5% CO₂, and culture supernatants were harvested 48 or 72 h after stimulation to measure TNF-α or IFN-γ, respectively.

4.10. Myeloperoxidase Activity

Five mice per group (C57BL/6 and IL-6 KO) were infected i.p. with 1 × 10⁶ virulent *B. abortus* in 100 mL of PBS. At 1 week post-infection, animals were sacrificed and their livers homogenized using a tissue homogenizer (T10 Basic ULTRA-TURRAX, IKA, Königswinter, Germany). Liver homogenates used to determine myeloperoxidase (MPO) activity, as previously described [49], as an indirect measure of neutrophil numbers [50]. In short, the livers were homogenized in 50 mM HEPES buffer (pH 8.0) and centrifuged, and the pellets were rehomogenized in H₂O/0.5% cetyltrimethylammonium chloride (CTAC) (Merck, Darmstadt, Germany). After centrifugation, supernatants were diluted in 10 mM citrate buffer, pH 5.0/0.22% CTAC, and substrate solution (3 mM 3,5,5-tetramethylbenzidine dihydrochloride (TMB) (Sigma), 120 M resorcinol (Merck), and 2.2 mM H₂O₂ in distilled water) was added. These reaction mixtures were incubated for 30 min at 37 °C and stopped by the addition of H₂SO₄. Optical density (OD) at 450 nM was determined.

4.11. Statistical Analysis

The results of this study were analyzed using two-way ANOVA, one-way ANOVA, or Student’s t-test, as specified in each figure legend, with GraphPad Prism 5 computer software (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at a p value < 0.05.

**Author Contributions:** Conceptualization, E.S.G., M.T.R.G., and S.C.O.; data curation, E.S.G., M.T.R.G., D.M.C., and J.M.M.; formal analysis E.S.G., M.T.R.G., and S.C.O.; funding acquisition, S.C.O.; investigation, E.S.G., M.T.R.G., J.M.M., and D.M.C.; methodology, E.S.G., M.T.R.G., J.M.M., and D.M.C.; project administration, S.C.O.; resources, S.C.O.; supervision, M.T.R.G. and S.C.O.; validation, S.C.O.; writing—original draft, E.S.G.; writing—review and editing, E.S.G., M.T.R.G., and S.C.O. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (grant nos. 302660/2015-1; 406883/2018-1), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) (grant nos. APQ-01945-17 and Rede Mineira de Imunobiologicos #00140-16), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior/Programa Nacional de Pós Doutorado CAPES/PNPD, and the National Institutes of Health (R01 AI116453).

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Hunter, A.C.; Jones, A.S. IL-6 as a keystone cytokine in health and disease. *Nat. Immunol.* 2015, 16, 448–457. [CrossRef] [PubMed]
2. Dinarello, C.A. The Endogenous Pyrogens in Host-Defense Interactions. *Hosp. Pr.* 1989, 24, 111–128. [CrossRef] [PubMed]
3. Tanaka, T.; Narazaki, M.; Kishimoto, T. IL-6 in Inflammation, Immunity, and Disease. *Cold Spring Harb. Perspect. Biol.* 2014, 6, a016295. [CrossRef] [PubMed]
4. Dann, S.M.; Spehlmann, M.E.; Hammond, D.C.; Imura, M.; Hase, K.; Choi, L.J.; Hanson, E.; Eckmann, L. IL-6-Dependent Mucosal Protection Prevents Establishment of a Microbial Niche for Attaching/Effacing Lesion-Forming Enteric Bacterial Pathogens. *J. Immunol.* 2008, 180, 6816–6826. [CrossRef]
5. Hack, C.E.; De Groot, E.R.; Felt-Bersma, R.J.; Nuijens, J.H.; Strack Van Schijndel, R.J.; Eerenberg-Belmer, A.J.; Thijs, L.G.; Aarden, L.A. Increased plasma levels of interleukin-6 in sepsis. *Blood* 1989, 74, 1704–1710. [CrossRef]
6. Ladel, C.H.; Blum, C.; Dreher, A.; Reifenberg, K.; Kopf, M.; Kaufmann, S.H. Lethal tuberculosis in interleukin-6-deficient mutant mice. * Infect. Immun.* 1997, 65, 4843–4849. [CrossRef]
11. Williams, D.M.; Grubbs, B.G.; Darville, T.; Kelly, K.; Rank, R.G. A role for interleukin-6 in host defense against murine Chlamydia trachomatis infection. Infect. Immun. 1998, 66, 4564–4567. [CrossRef] [PubMed]

12. Saunders, B.M.; Frank, A.A.; Orme, I.M.; Cooper, A.M. Interleukin-6 Induces Early Gamma Interferon Production in the Infected Lung but Is Not Required for Generation of Specific Immunity to Mycobacterium tuberculosis Infection. Infect. Immun. 2000, 68, 3322–3326. [CrossRef] [PubMed]

13. Franco, M.P.; Mulder, M.; Gilman, R.H.; Smits, H.L. Human brucellosis. Lancet Infect. Dis. 2007, 7, 775–786. [CrossRef]

14. Oliveira, S.C.; Giambartolomei, G.H.; Cassataro, J. Confronting the barriers to develop novel vaccines against brucellosis. Expert Rev. Vaccines 2011, 10, 1291–1305. [CrossRef]

15. Oliveira, S.C.; De Oliveira, F.S.; Macedo, G.C.; De Almeida, L.A.; Carvalho, N.B. The role of innate immune receptors in the control of Brucella abortus infection: Toll-like receptors and beyond. Microbes Infect. 2008, 10, 1005–1009. [CrossRef]

16. Brandão, A.P.M.S.; Oliveira, F.S.; Carvalho, N.B.; Vieira, L.Q.; Azevedo, V.; Macedo, G.C.; Oliveira, S.C. Host Susceptibility to Brucella abortus Infection Is More Pronounced in IFN-γ knock-out than IL-12/β2-Microglobulin Double-Deficient Mice. Clin. Dev. Immunol. 2012, 2012, 1–7. [CrossRef]

17. Gomes, M.T.R.; Campos, P.C.; De Almeida, L.A.; Oliveira, F.S.; Costa, M.M.S.; Marim, F.M.; Pereira, G.S.M.; Oliveira, S.C. The role of innate immune signals in immunity to Brucella abortus. Front. Cell. Infect. Microbiol. 2012, 2, 130. [CrossRef]

18. Zhan, Y.; Liu, Z.; Cheers, C. Tumor necrosis factor alpha and interleukin-12 contribute to resistance to the intracellular bacterium Brucella abortus by different mechanisms. Infect. Immun. 1996, 64, 2782–2786. [CrossRef]

19. Dalrymple, S.A.; Lucian, L.A.; Slattery, R.; McNeil, T.; Aud, D.M.; Fuchino, S.; Lee, F.; Murray, R. Interleukin-6-deficient mice are highly susceptible to Listeria monocytogenes infection: Correlation with inefficient neutrophilia. Infect. Immun. 1995, 63, 2262–2268. [CrossRef]

20. Dalrymple, S.A.; Slattery, R.; Aud, D.M.; Krishna, M.; ALucian, L.; Murray, R. Interleukin-6 is required for a protective immune response to systemic Escherichia coli infection. Infect. Immun. 1996, 64, 3231–3235. [CrossRef]

21. Cerqueira, D.M.; Gomes, M.T.R.; Silva, A.L.N.; Rungue, M.; Assis, N.R.G.; Guimarães, E.S.; Morais, S.B.; Broz, P.; Zamboni, D.S.; Oliveira, S.C. Guanylate-binding protein 5 licenses caspase-11 for Gasdermin-D mediated host resistance to Brucella abortus infection. PLoS Pathog. 2018, 14, e1007519. [CrossRef] [PubMed]

22. Dube, P.H.; Handley, S.A.; Lewis, J.; Miller, V.L. Protective Role of Interleukin-6 during Yersinia enterocolitica Infection Is Mediated through the Modulation of Inflammatory Cytokines. Infect. Immun. 2004, 72, 3561–3570. [CrossRef] [PubMed]

23. Chalaris, A.; Garbers, C.; Rabe, B.; Rose-John, S.; Scheller, J. The soluble Interleukin 6 receptor: Generation and role in inflammation and cancer. Eur. J. Cell Biol. 2011, 90, 484–494. [CrossRef] [PubMed]

24. Scheller, J.; Chalaris, A.; Schmidt-Arras, D.; Rose-John, S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim. Biophys. Acta (BBA) Mol. Cell Res. 2011, 1813, 878–888. [CrossRef]

25. Jiang, X.; Baldwin, C.L. Effects of cytokines on intracellular growth of Brucella abortus. Infect. Immun. 1993, 61, 124–134. [CrossRef]
26. Murphy, E.A.; Sathiyaseelan, J.; Parent, M.A.; Zou, B.; Baldwin, C.L. Interferon-gamma is crucial for surviving a *Brucella abortus* infection in both resistant C57BL/6 and susceptible BALB/c mice. *Immunol.* 2001, 103, 511–518. [CrossRef]

27. Zhan, Y.; Cheers, C. Endogenous gamma interferon mediates resistance to *Brucella abortus* infection. *Infect. Immun.* 1993, 61, 4899–4901. [CrossRef]

28. Bradley, L.M.; Dalton, D.K.; Croft, M. A direct role for IFN-gamma in regulation of Th1 cell development. *J. Immunol.* 1996, 157, 1350–1358.

29. Tau, G.; Rothman, P.B. Biologic functions of the IFN-gamma receptors. *Allergy* 1999, 54, 1233–1251. [CrossRef]

30. Fuster, J.J.; Walsh, K. The Good, the Bad, and the Ugly of interleukin-6 signaling. *EMBO J.* 2014, 33, 1425–1427. [CrossRef]

31. Lin, Z.Q.; Lin, G.Y.; He, W.W.; Zhang, C.; Zhang, R.; Li, Y.D.; Wang, F.; Qin, Y.; Duan, L.; Zhao, D.-D.; et al. IL-6 and INF-γ levels in patients with brucellosis in severe epidemic region, Xinjiang, China. *Infect. Dis. Poverty* 2020, 9, 47. [CrossRef] [PubMed]

32. Hop, H.T.; Huy, T.X.N.; Reyes, A.W.B.; Arayan, L.T.; Vu, S.H.; Min, W.; Lee, H.J.; Kang, C.K.; Kim, D.H.; Tark, D.S.; et al. Interleukin 6 Promotes *Brucella abortus* Clearance by Controlling Bacterial Activity of Macrophages and CD8+ T Cell Differentiation. *Infect. Immun.* 2019, 87. [CrossRef] [PubMed]

33. Heremans, H.; Dillen, C.; Put, W.; Van Damme, J.; Billiau, A. Protective effect of anti-interleukin (IL)-6 antibody against endotoxin, associated with paradoxically increased IL-6 levels. *Eur. J. Immunol.* 1992, 22, 2395–2401. [CrossRef] [PubMed]

34. Lu, Z.Y.; Brochier, J.; Wijdenes, J.; Brailly, H.; Bataille, R.; Klein, B. High amounts of circulating interleukin (IL)-6 in the form of monomeric immune complexes during anti-IL-6 therapy. Towards a new methodology for measuring overall cytokine production in humanin vivo. *Eur. J. Immunol.* 1992, 22, 2819–2824. [CrossRef]

35. May, L.T.; Neta, R.; Moldawer, L.L.; Kenney, J.S.; Patel, K.; Sehgal, P.B. Antibodies chaperone circulating IL-6. Paradoxical effects of anti-IL-6 “neutralizing” antibodies in vivo. *J. Immunol.* 1993, 151, 3225–3236.

36. Romani, L.; Mencacci, A.; Cenci, E.; Spaccapelo, R.; Toniatti, C.; Puccetti, P.; Bistoni, F.; Poli, V. Impaired neutrophil response and CD4+ T helper cell 1 development in interleukin 6-deficient mice infected with Candida albicans. *J. Exp. Med.* 1996, 183, 1345–1355. [CrossRef]

37. Roset, M.S.; Ibañez, A.E.; Filho, J.A.D.S.; Spera, J.M.; Minatel, L.; Oliveira, S.C.; Giambartolomei, G.H.; Cassataro, J.; Briones, G. Brucella Cyclic β-1,2-Glucan Plays a Critical Role in the Induction of Splenomegaly in Mice. *PLoS ONE* 2014, 9, e101279. [CrossRef]

38. Fielding, C.A.; McLoughlin, R.M.; McLeod, L.; Colmont, C.S.; Najdovska, M.; Grail, D.; Ernst, M.; Jones, S.A.; Topley, N.; Jenkins, B.J. IL-6 Regulates Neutrophil Trafficking during Acute Inflammation via STAT3. *J. Immunol.* 2008, 181, 2189–2195. [CrossRef]

39. Oshshima, S.; Saeki, Y.; Mima, T.; Sasai, M.; Nishioka, K.; Nomura, S.; Kopf, M.; Katada, Y.; Tanaka, T.; Suemura, M.; et al. Interleukin 6 plays a key role in the development of antigen-induced arthritis. *Proc. Natl. Acad. Sci. USA* 1998, 95, 8222–8226. [CrossRef]

40. Yamamoto, M.; Yoshizaki, K.; Kishimoto, T.; Ito, H. IL-6 Is Required for the Development of Th1 Cell-Mediated Murine Colitis. *J. Immunol.* 2000, 164, 4878–4882. [CrossRef]

41. Ulich, T.R.; del Castillo, J.; Guo, K.Z. In vivo hematologic effects of recombinant interleukin-6 on hematopoiesis and circulating numbers of RBCs and WBCs. *Blood* 1989, 73, 108–110. [CrossRef] [PubMed]

42. Kaplanski, G. IL-6: A regulator of the transition from neutrophil to monocyte recruitment during inflammation. *Trends Immunol.* 2003, 24, 25–29. [CrossRef]

43. Romano, M.; Sironi, M.; Toniatti, C.; Polentarutti, N.; Fruscella, P.; Ghezzi, P.; Faggioni, R.; Luini, W.; Van Hinsbergh, V.; Sozzani, S.; et al. Role of IL-6 and Its Soluble Receptor in Induction of Chemokines and Leukocyte Recruitment. *Immunity* 1997, 6, 315–325. [CrossRef]

44. McLoughlin, R.M.; Witowski, J.; Robson, R.L.; Wilkinson, T.S.; Hurst, S.M.; Williams, A.S.; Williams, J.D.; Rose-John, S.; Jones, S.A.; Topley, N. Interplay between IFN-γ and IL-6 signaling governs neutrophil trafficking and apoptosis during acute inflammation. *J. Clin. Invest.* 2003, 112, 598–607. [CrossRef] [PubMed]

45. Biffi, W.L.; Moore, E.E.; Moore, F.A.; Barnett, C.C.; Carl, V.S.; Peterson, V.M. Interleukin-6 Delays Neutrophil Apoptosis. *Arch. Surg.* 1996, 131, 24–30. [CrossRef]
46. Gomes, M.T.R.; Campos, P.C.; Oliveira, F.S.; Corsetti, P.P.; Bortoluci, K.R.; Cunha, L.D.; Zamboni, D.S.; Oliveira, S.C. Critical Role of ASC Inflammasomes and Bacterial Type IV Secretion System in Caspase-1 Activation and Host Innate Resistance to Brucella abortus Infection. *J. Immunol.* 2013, 190, 3629–3638. [CrossRef]

47. Guimarães, E.S.; Gomes, M.T.R.; Campos, P.C.; Mansur, D.S.; Santos, A.A.; Harms, J.; Splitter, G.; Smith, J.A.; Barber, G.N.; Oliveira, S.C. *Brucella abortus* Cyclic Dinucleotides Trigger STING-Dependent Unfolded Protein Response That Favors Bacterial Replication. *J. Immunol.* 2019, 202, 2671–2681. [CrossRef]

48. Gomes, M.T.; Campos, P.C.; Pereira, G.D.S.; Bartholomeu, D.C.; Splitter, G.; Oliveira, S.C. TLR9 is required for MAPK/NF-κB activation but does not cooperate with TLR2 or TLR6 to induce host resistance to *Brucella abortus*. *J. Leukoc. Biol.* 2015, 99, 771–780. [CrossRef]

49. Eijkelkamp, W.B.; Zhang, Z.; Remuzzi, G.; Parving, H.-H.; Cooper, M.E.; Keane, W.F.; Shahinfar, S.; Gleim, G.W.; Weir, M.R.; Brenner, B.M.; et al. Albuminuria Is a Target for Renoprotective Therapy Independent from Blood Pressure in Patients with Type 2 Diabetic Nephropathy: Post Hoc Analysis from the Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) Trial. *J. Am. Soc. Nephrol.* 2007, 18, 1540–1546. [CrossRef]

50. Pulli, B.; Ali, M.; Forghani, R.; Schob, S.; Hsieh, K.L.C.; Wojtkiewicz, G.; Linnoila, J.J.; Chen, J.W. Measuring Myeloperoxidase Activity in Biological Samples. *PLoS ONE* 2013, 8, e67976. [CrossRef]

**Publisher’s Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations. 

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).