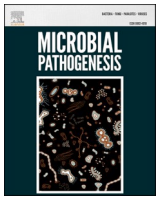
Microbial Pathogenesis 164 (2022) 105458

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath



Platelet depletion does not alter the course of *Brucella abortus* infection *in vivo*

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ARTICLE INFO

Keywords:
Platelets
Aspercetin
Brucella abortus
Mice
Depletion

ABSTRACT

Brucellosis is a bacterial disease of animals and a zoonotic infection. Thrombocytopenia is a common outcome in long-lasting brucellosis in humans. Likewise, *ex vivo* experiments have shown that platelets may play a role in *Brucella abortus* infections. Following these reports, we explored the course of brucellosis in thrombocytopenic mice, using the non-toxic low-molecular-weight aspercetin protein that depletes platelets *in vivo*. Aspercetin does not induce systemic hemorrhage or inflammation, and when injected into mice, it generates a rapid dose-dependent drop in platelet counts without affecting central organs, disrupting hematological parameters, or the proinflammatory cytokine profile. Compared to the *B. abortus* infected control group, the infected thrombocytopenic mice did not show significant differences in the hematological profiles, pathological score, spleen, liver histopathology, or bacterial loads. Except for IL-6, which was higher in the infected thrombocytopenic mice, the TNF-α, IFN-γ and IL-10 did not significantly differ with the PBS-infected group. The results indicate that platelets do not play a significant role in modulating *Brucella* infection *in vivo* at the early stages of infection, which is commensurate with the stealthy strategy followed by *Brucella* organisms at the onset of the disease.

1. Introduction

Members of the genus *Brucella* are bacterial pathogens of domestic animals and a source of human infections [1]. *Brucella abortus*, displaying a preference for bovine hosts, is one of the most zoonotic and economically relevant species [2]. This bacterium is a fine-tuned intracellular pathogen that displays a furtive and stealthy strategy at early times of the infection [3], with the participation of elements of the immune response as modulators [4]. The lag between the innate and adaptive immune responses allows the bacterium to disperse and invade different tissues before the latter response becomes fully activated [3,4]. We have shown that neutrophils (PMNs) and complement depletion enhance the immune response in mice with brucellosis, eliminating the bacterium from the target organs more efficiently than infected mice non-depleted of these two components of the innate immune response [5,6).

Platelets are cellular elements capable of modulating proinflammatory and immune processes (7,8]; by releasing molecular mediators that interact with cells and components of the innate and acquired immunity. Due to their high number of circulating platelets and their ability to release proinflammatory mediators, these elements have been considered "sentinels" capable of communicating with immune cells. For instance, when platelets are activated, they express on their surface P-selectin, an adhesion molecule that binds the glycoprotein called PSGL-1 found on monocytes, PMNs, and lymphocytes [9]. As a result, these cells can form complexes with platelets and modify their rolling and arrest on the adherent surface, thus inducing vascular proinflammation. Moreover, platelets express Toll-like receptors (TLRs) linking the innate with the adaptive immune response during infectious and proinflammatory processes [10].

At the onset and early stages of the infection, hematological changes are not conspicuous in brucellosis [11]. However, among the most

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https://doi.org/10.1016/j.micpath.2022.105458
Received 12 January 2022; Received in revised form 22 February 2022; Accepted 23 February 2022
Available online 25 February 2022

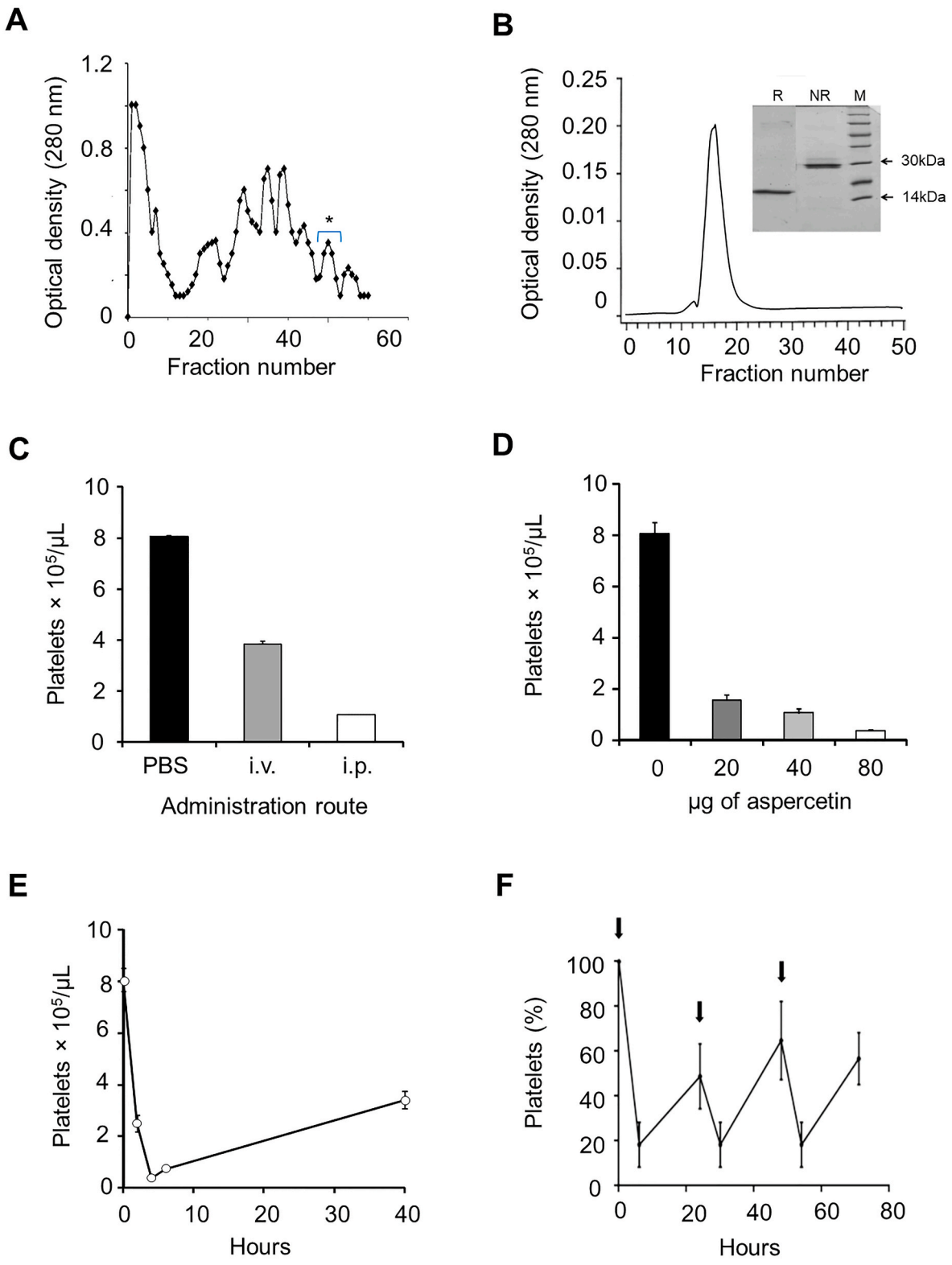

Fig. 1. Isolation of aspercetin and counts of platelets after depletion (A) DEAE-Sepharose fractionation of *B. asper* venom (500 mg) using 0.01 M phosphate buffer and linear NaCl gradient (0–0.3 M). Aspercetin is found in fraction label with "*". (B) Subsequent purification of reach aspercetin fraction from "A" in Affi-Gel Blue equilibrated with 0.015 M Tris buffer, pH 8.0, and A linear NaCl gradient (0–1.5 M). The purity of aspercetin fraction was analyzed by SDS-PAGE in 12% acrylamide gel, under reducing (R) and non-reducing (NR) conditions; molecular weight markers (M). (C) platelets numbers were estimated at 24 h after mice received 40 μg of aspercetin by intravenous (i.v.) or intraperitoneal (i.p.) routes. Control mice received PBS by i.v or i.p routes, with no difference (D). The number of platelets was estimated at 24 h in mice receiving different doses of aspercetin by the i.p. route. (E) Over time, the number of platelets was estimated in mice after receiving 80 μg by the i.p. route. (F) The number of platelets was quantified at different time points after consecutive administration of 80 μg of aspercetin i.v. (black arrows) at 0, 24, and 48 h. Bars represent the standard error.

Table 1
Hematological values of CD1 mice after 6 h of i.p administration of PBS or 80 μg of aspercetin.

Parameter	PBS			Aspercetin		
	Unit	Mean	CV[a] 95%	Mean	CV[a] 95%	*p* value
Leucocytes	10^9/L	5.90	[4.13–7.67]	7.55	[5.09–10.01]	0.31
Lymphocytes	10^9/L	3.75	[2.37–5.13]	5.28	[3.42–7.14}	0.22
Monocytes	10^9/L	0.20	[0.06–0.34]	0.33	[0.00–0.69]	0.53
Neutrophils	10^9/L	1.51	[0.90–2.12]	1.94	[1.24–2.64]	0.38
Lymphocytes	%	67.07	[60.18–73.96]	69.16	[64.32–74.00]	0.64
Monocytes	%	4.33	[1.04–7.62]	3.84	[0.77–6.91]	0.84
Neutrophils	%	28.60	[20.48–36.72]	26.99	[21.00–32.98]	0.76
Erythrocytes	10^{12}/L	5.95	[5.33–6.57]	6.55	[5.83–7.27]	0.24
Hemoglobin	g/dL	8.23	[7.20–9.26]	9.24	[8.23–10.25]	0.19
Hematocrit	%	27.81	[24.33–31.29]	28.99	[25.83–32.15]	0.63
Platelets[b]	10^9/L	682.14	[543.28–821.00]	168	[122.34–213.66]	0.0002

[a] CV, coefficient of variation.
[b] Mean platelet volume (MPV) ranged from 6.32 to 9.00 fL with no significant differences between groups.

common hematological clinical features of long-lasting human brucellosis is the significant reduction in circulating platelets, which is reestablished to normal values after patients are subjected to antibiotics treatment [12]. Like other bacterial infections, these events suggest that platels may also play a role in brucellosis [13]. In *ex vivo* experiments, it has been shown that *Brucella* organisms are capable of interacting and inducing the activation of platelets, and when these cellular elements are incubated with either macrophages or PMNs, they promote their activation [14,15]. Although platelets did not modify the initial invasion of *B. abortus* to macrophages and PMNs, their presence improved the control of these leukocytes to bacterial infection several hours later, suggesting that although plates do not modulate the bactericidal function of these cells at the onset of the infection, they are capable of doing it at later times. Likewise, *B. abortus* macrophage infection induces secretion of platelet-activating factor (PAF), which promotes the phagocytosis of this bacterium associated with the activation of the Janus kinase 2 (JAK2) [16].

Following this, we have explored the function of platelets *in vivo*, using a murine thrombocytopenic model to determine the influence of these cellular elements in the course of infection in mice. Overall, our results demonstrate that the sustained severe reduction of circulating platelets did not alter the course of *B. abortus* infection in the murine model.

2. Material and methods

2.1. Aspercetin isolation and purification

Aspercetin was obtained as previously described [17]. Briefly, *Bothrops asper* venom (500 mg) was fractioned using DEAE-sepharose and 0.01 M phosphate buffer (pH 7.8) and linear NaCl gradient (0–0.3 M). The aspercetin fraction was further purified by affinity chromatography on Affi-Gel Blue. Next, the aspercetin fraction was visualized by an electrophoretic analysis (SDS-PAGE) using 12% acrylamide gel. Finally, aspercetin was lyophilized for further usage.

2.2. Mice infection and platelet depletion protocols

B. abortus 2308 W was used in all experiments. CD1 mice were supplied by the Escuela de Medicina Veterinaria, Universidad Nacional, and Instituto Clodomiro Picado Twight, Universidad de Costa Rica. Female mice (18–22 g) were housed under specific pathogen-free conditions, kept in cages with food and water *ad libitum*. Animal experimentation was conducted as defined by "Comite' Institucional para el cuido y uso de los animales'' of the Universidad Nacional and following the guidelines and consent of the "Comité Institucional para el Cuido y Uso de los Animales de la Universidad de Costa Rica" (CICUA-067-17) and in accordance with the corresponding Animal Welfare Law of Costa Rica (Law 9458). All animals were kept in cages with food and water *ad libitum* under biosafety containment conditions. For platelet depletion, purified aspercetin (80 μg) was administered to mice at 0, 24, and 48 h through the intraperitoneal (i.p.) route. PBS or aspercetin treated mice were infected after 20 h of first aspercetin or PBS injection by the intraperitoneal route (i.p.) with 10^6 colony forming units (CFU) of *B. abortus,* and spleen weights and bacteria numbers were counted as described elsewhere [18].

2.3. Histopathology, hematological profiles, and cytokine quantification

For histopathological studies, the spleen and liver were fixed in 10% neutral buffered formalin, processed, and stained with hematoxylin and eosin stain [18]. The lesions were determined by semiquantitative analysis and scored as negative (0) to severe (4) as previously described [19,20]. Quantitative determination of TNF-alpha, IFN-gamma, IL-6, and IL-10, the serum from mice were collected and the concentration of cytokines measured by ELISA according to the manufacturer's specifications (Invitrogen), as described [18,19]. A complete hematological analysis and platelet counts in the blood of mice (collected in EDTA) were performed manually in a hemocytometer under the microscope or automated in a VetScan HM5 Hematology Analyzer, following the manufacturer's instructions.

2.4. Statistics

One-way analysis of variance (ANOVA) followed by Dunnett's test or multivariate analysis of variance (MANOVA) was used to determine statistical significance in the different assays. The JMP (https://www.jmp.com) and GraphPad Prism software (version 8.0.1) (https://www.graphpad.com) was used for statistical analysis. Data were processed in Microsoft Office Excel 365.

3. Results

After chromatography (Fig. 1A), the high purity of aspercetin was revealed by the presence of one band in SDS-PAGE using reducing and non-reducing conditions (Fig. 1B), confirming previous results [17]. To evaluate the best route of aspercetin administration, we estimated the number of platelets after the intravenous (i.v.) or intraperitoneal (i.p.) administration of 40 μg of aspercetin. We found that platelet depletion was best achieved by the i.p route (Fig. 1C). Then, we assessed the optimal dose of aspercetin by the i.p. route in time. The best results were obtained with 80 μg/mouse following the i.p route (Fig. 1D). Higher doses of aspercetin induced sustained hemorrhage, mainly at the injection site, and therefore not evaluated. Finally, we estimated the number of platelets over time after one dose of 80 μg i.p. The number of platelets reached the minimum number at 4 h post-treatment, and then

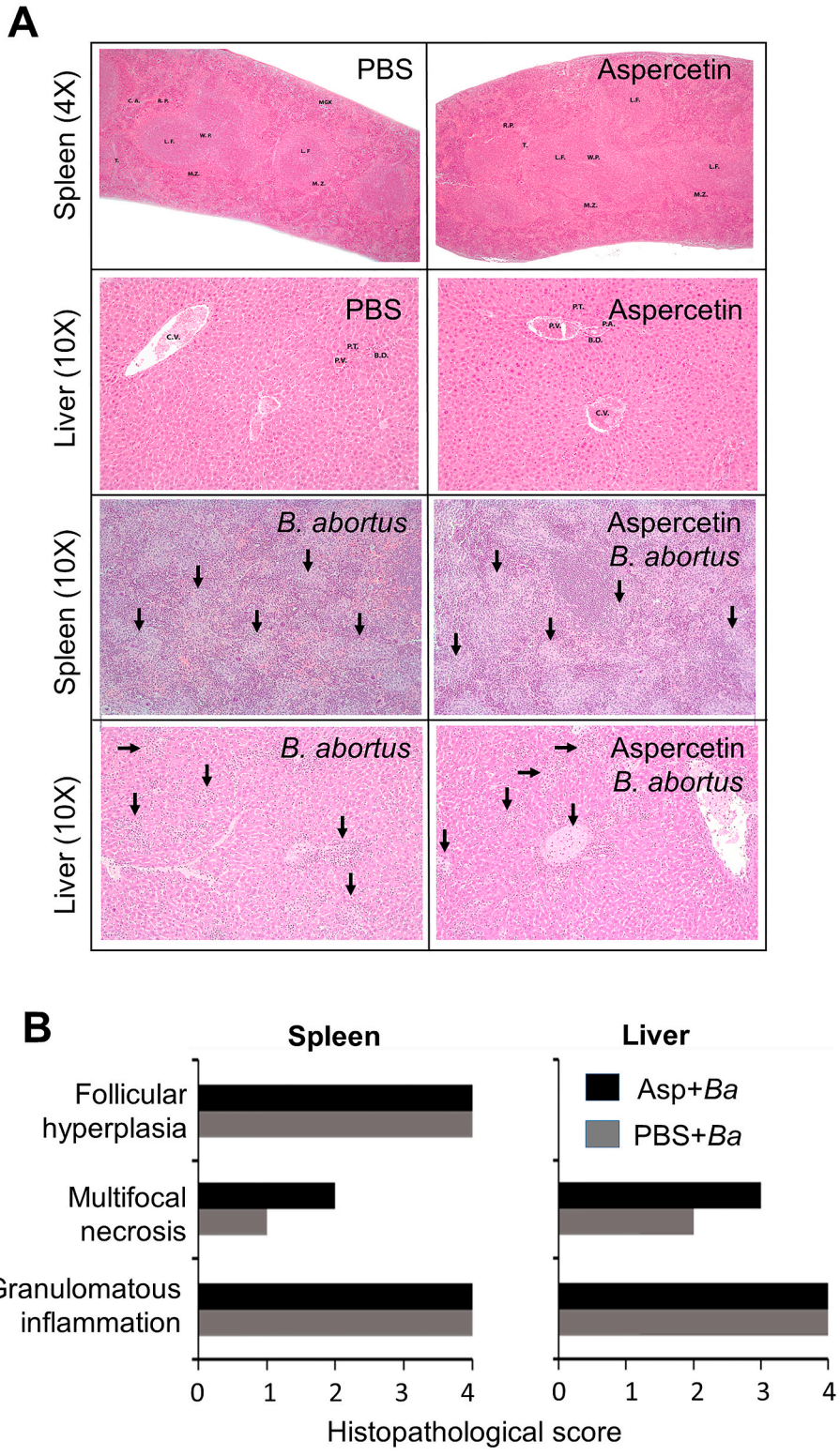

Fig. 2. Histopathological analysis of *B. abortus* thrombocytopenic infected mice. (A) Mice were treated with PBS or 80 μg of aspercetin i.p. at 0, 24, and 48 h: then either infected (at the time "24") or not with 10^6 of *B. abortus*. Mice were then sacrificed at day seven post-treatment and post-infection, and sections of the spleen and liver were analyzed by histopathological examination after being stained with hematoxylin-eosin. (A) The histopathological score was evaluated in hematoxylin eosin-stained sections of spleen and liver of no-infected and *B. abortus* infected mice. Black arrows indicate the granulomas in the spleen and liver of *B. abortus*-infected mice.

Table 2
Hematological values of CD1 mice at seven days post-infection with *B. abortus* previously treated with PBS or aspercetin.

Parameter		PBS + *B. abortus*		Aspercetin + *B. abortus*		
	Unit	Mean	CV 95%[a]	Mean	CV 95%	*p* value
Total leucocytes	10^9/L	9.48	[6.12–12.85]	10.73	[8.30–13.17]	0.52
Lymphocytes	10^9/L	5.27	[3.34–7.20]	5.70	[4.05–7.36]	0.71
Monocytes	10^9/L	0.35	[0.20–0.50]	0.49	[0.29–0.70]	0.23
Neutrophils	10^9/L	3.86	[2.46–5.25]	4.54	[3.60–5.47]	0.39
Lymphocytes	%	55.20	[49.96–60.44]	51.87	[47.13–56.60]	0.31
Monocytes	%	3.59	[2.62–4.57]	5.15	[2.66–7.64]	0.22
Neutrophils	%	41.18	[36.03–46.34]	43.00	[39.59–46.41]	0.53
Erythrocytes	10^{12}/L	6.38	[5.83–6.93]	6.22	[5.55–6.87]	0.68
Hemoglobin	g/dL	8.68	[7.66–9.71]	8.52	[7.13–9.90]	0.83
Hematocrit	%	32.01	[27.63–36.38]	31.71	[26.24–37.17]	0.93
Platelets[b]	10^9/L	494.3	[392.5–596.2]	600.6	[486.4–714.8]	0.14

[a] CV, coefficient of variation.
[b] Mean platelet volume (MPV) ranged from 6.46 to 7.45 fL with no significant differences between groups.

the number slowly increased over time (Fig. 1E). To keep mice under thrombocytopenia, we administered 80 μg of aspercetin, i.p., every 24 h for three days. As shown in Fig. 1F, aspercetin quickly decreased the platelet's proportion when administrated repeatedly.

We then verified whether continuous administration of aspercetin affected the hematological profile or generated any histologic lesions to the spleen and liver of mice. As observed in Table 1 the only parameter that showed a significant difference between the control (PBS) and the aspercetin treated mice groups was the low platelet count in the latter. However, the mean platelet volume (MPV) remained within normal parameters in both groups (6.32–9.00 fL). Likewise, the spleen and liver of these two groups of mice showed unmarked structural and histopathological characteristics after seven days of three consecutive doses (0 h, 24 h, and 48 h) of aspercetin (Fig. 2A).

Then we explored the effect of *B. abortus* on thrombocytopenic mice treated with aspercetin. As expected, both infected groups showed substantial changes in the hematological profile after one week post-infection (Table 2). However, no significant differences were observed between the infected control group and the infected thrombocytopenic mice. However, platelets were significantly lower in the infected mice than the normal non-infected mice ($p < 0.05$) (Tables 1 and 2). Moreover, the MPV values of both the control and aspercetin treated infected mice were not different at this time point (6.46–7.45 fL). Likewise, the spleen and liver of control and thrombocytopenic mice infected with *B. abortus* showed very similar granulomatous inflammatory and necrotic lesions in both the spleen and liver, characteristic of murine brucellosis (Fig. 2A). The histopathological score remained similar in both groups, with slightly higher multifocal necrosis in the organs of the thrombocytopenic *B. abortus*-infected mice (Fig. 2B).

Repeated treatment with aspercetin at 0, 24, and 48 h did not increase the spleen inflammation (weight) after seven days (Fig. 3A). In contrast, the weights of the spleens of *B. abortus* infected mice increased considerably, with no significant difference between the thrombocytopenic and the PBS groups. Similarly, no significant difference in the bacterial loads was observed between both groups of mice infected with *B. abortus* (Fig. 3B). Concomitant to these results, we did not observe differences in the TNFα, INF-γ and IL-10 response between the normal and thrombocytopenic infected mice. However, a significant difference was observed in the IL-6 response between these two groups of mice (Fig. 4).

4. Discussion

Although we did not compare our system with other methods, our aspercetin platelet depletion model seems adequate and at least comparable to other models. Aspercetin is a disulfide-linked heterodimer of 29,759 Da belonging to the C-type lectin family that induces platelet aggregation in the presence of von Willebrand factor via the receptor GPIb [17]. This small protein does not interact with blood cells, lacks an anticoagulant effect, and is not lethal. Moreover, aspercetin does not induce systemic hemorrhage or inflammation. When injected into mice, it generates a rapid dose-dependent drop in platelet counts and prolongs the bleeding time. However, it does not affect the central organs such as the spleen, liver, and lungs, nor does it disrupt the hematological profile, the MPV, or the proinflammatory cytokine profile, as demonstrated here. After a single injection, our aspercetin model achieved depletion dawn to 20,000 platelets/μL after single injection. However, in general, mice are resilient to an antibody or aspercetin platelet depletion, and the numbers steadily increase over time, requiring subsequent injections of the depleting agent to keep the thrombocytopenic stage. Because of its properties, aspercetin constitutes a good alternative for generating a chronic thrombocytopenic stage in animal models for evaluating the function of platelets, as demonstrated before [21].

Thrombocytopenic mouse models have been used to unveil the role of platelets against several bacterial infections. For instance, antibody depletion of platelets in *Klebsiella*-infected mice was associated with increased bacterial growth in lungs, blood, and distant organs and impaired survival during pneumonia-derived sepsis [22]. The signs of *Klebsiella*-induced disease were proportional to the platelet depletion level, also associated with enhanced cytokine release. Likewise, in a diphtheria-toxin mediated megakaryocyte depletion model, mice succumbed to *Staphylococcus aureus* infection more rapidly, contained significantly higher bacterial burden in the target organs, and produced higher cytokine levels than control mice [23]. *Pseudomonas aeruginosa* antibody-depleted infected mice displayed a significant reduction in the recruitment of PMNs, and an increased systemic bacterial dissemination and mortality rates compared with non-platelet-depleted control mice [24]. The aspercetin induced thrombocytopenic mice model has also been used in infection protocols. For instance, it was shown that platelet-depleted mice with aspercetin were unable to control the lymphocytic choriomeningitis virus in mice inducing generalized splenic

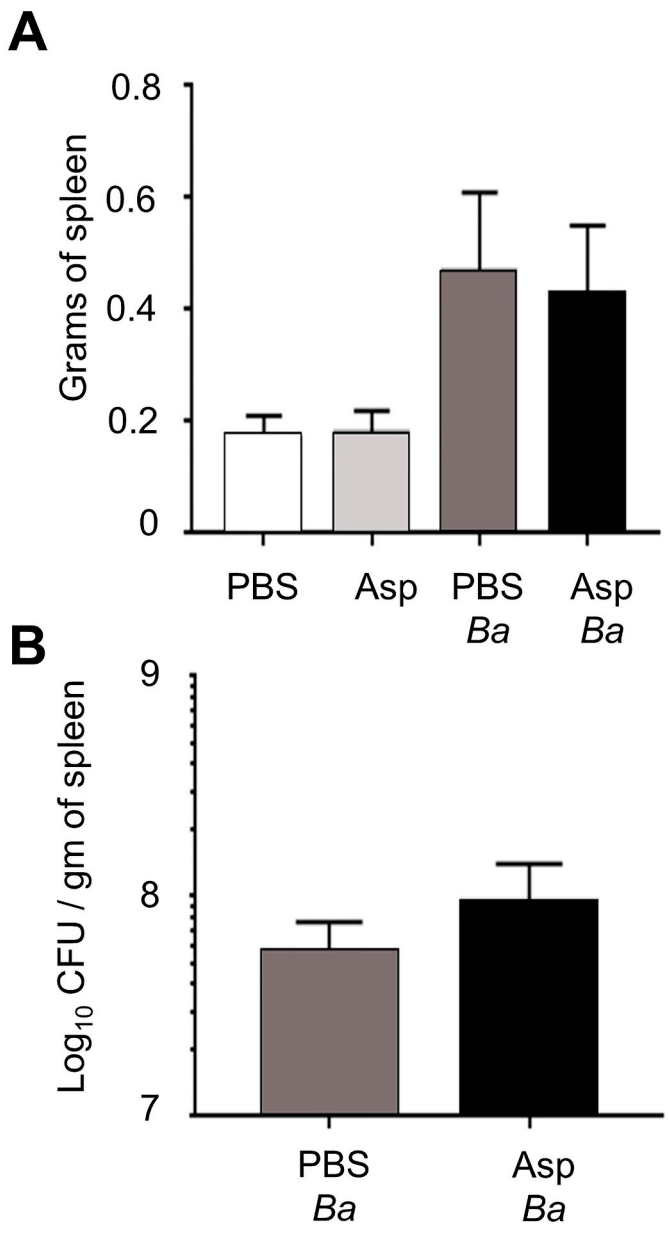

Fig. 3. Spleen weights and bacterial counts of *B. abortus* infected thrombocytopenic mice. Mice were treated with PBS or 80 μg of aspercetin i.v., at 0, 24, and 48 h. Mice were infected with 106 UFC of *B. abortus* 2308 at h "24" concomitant with the second dose of aspercetin. Mice were sacrificed seven days post-infection. (A) Spleen weights were recorded for every group, and (B) bacterial burden was determined for the infected groups. Bars represent the error standard.

necrosis, affecting the innate and adaptive immune response [21].

Following this, it is remarkable that in contrast to other microbial infections, the proinflammatory signs, hematological profiles, pathological characteristics, and bacterial burden of the thrombocytopenic *Brucella*-infected mice remain very similar to those infected mice with a complete platelet repertoire. Compared to other Gram-negative bacteria such as *Salmonella*, the *Brucella* organisms barely induce thrombocytopenia, coagulopathies, or other associated hematological changes at the onset of infection [3]. In addition, like platelets, other elements of the innate immune system such as PMNs and complement are also dispensable for developing an efficient immune defense against brucellosis [5,18], revealing a common trait of *Brucella* organisms concerning the elements of the innate immunity during infection.

However, thrombocytopenia is a frequent characteristic in long-lasting *Brucella* infections in humans, and mice, as revealed here (e.g., compare values of Tables 1 and 2) and in other works [12]. Thrombocytopenia as a complication of *Brucella* infection may result from multiple mechanisms, such as hypersplenism, bone marrow invasion, hemophagocytosis, or immune suppression due to the rise of antiplatelet antibodies, all events observed in brucellosis [25–27]. An interesting phenomenon probably linked to the thrombocytopenia in brucellosis may be the rise of IL-6. Indeed, with the sole exception of IL-6, which was more augmented in the thrombocytopenic *B. abortus* infected mice, all the other cytokines levels were comparable with normal mice infected with *B. abortus*. As shown here, it has been repeatedly demonstrated that the levels of IL-6 always increase after seven days during *Brucella* infections, and these bacteria readily invade bone marrow cells, establishing long-lasting infections in this tissue, promoting hemophagocytosis [27]. Therefore, the comparable higher levels of IL-6 in *B. abortus*-infected thrombocytopenic mice are remarkable since this cytokine induces pleiotropic effects on inflammation, immune response, and hematopoiesis, including megakaryopoiesis. For instance, it has been shown that IL-6 reaches the bone marrow, promoting megakaryocyte maturation [28] and that this cytokine also promotes the extracellular megakaryopoiesis in the spleen [29], thus leading to the release of platelets. In mice infected with brucellosis, extracellular megakaryopoiesis in the spleen and liver is a common outcome [30]. It may be that the presence of these bacteria comparatively increases even more the levels of IL-6 in thrombocytopenic mice as a compensation effect to promote the synthesis of platelets. Although the IL-6 increases above expected levels in the infected thrombocytopenic mice, its augmented values are not overly reflected in the proinflammatory response and immunity in murine brucellosis.

In contrast to long-lasting human brucellosis cases [31], we did not observe altered MPV values at the recorded times. The smaller platelet size results from faster impute of platelets into the circulation to compensate for the thrombocytopenia state. Consequently, smaller platelets may be generated at later stages of the *Brucella* infection in mice. Alternatively, the extramedullary megakaryopoiesis observed in *Brucella* infected mice infected may compensate for this phenomenon, in contrast to humans.

Altogether, our results indicate that platelets do not play a significant role in modulating the level of *Brucella* infection *in vivo* at early stages of infection, a fact that is commensurate with the stealthy strategy followed by *Brucella* organisms at the onset of the infection, which in the course is related to the absence of marked Pathogen-Associated Molecular Patterns in these bacteria [3].

CRediT authorship contribution statement

Berny Arias-Gómez: Investigation. **Roger Fonseca-Muñoz:** Investigation. **Alejandro Alfaro-Alarcón:** Investigation. **Carlos Chacón-Díaz:** Resources, Methodology. **Edgardo Moreno:** Visualization, Writing – original draft, Writing – review & editing, Conceptualization,

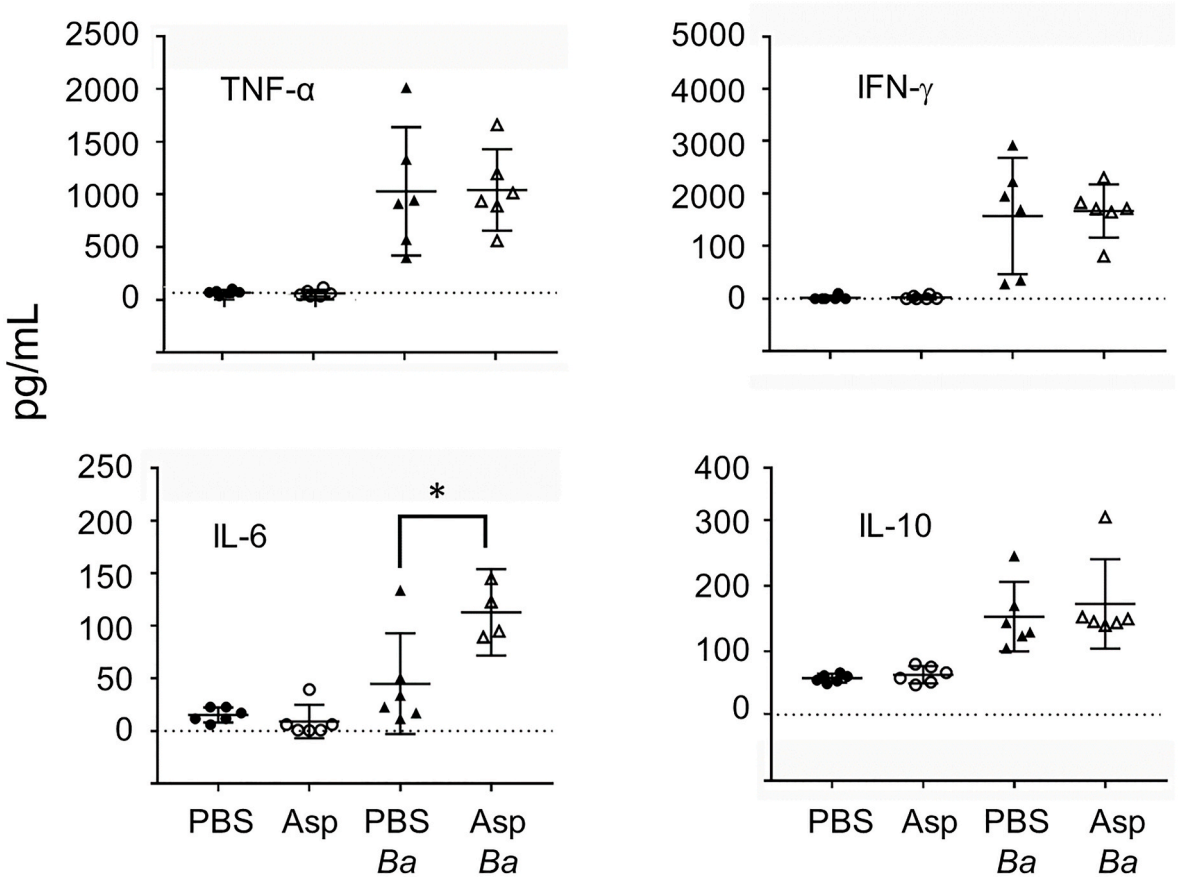

Fig. 4. Cytokines in *B. abortus* thrombocytopenic infected mice. We determined by ELISA the levels of de TNF-α, INF-γ, IL-6 e IL-10 in mice treated with PBS or 80 μg of aspercetin i.v., at 0, 24, and 48 h and infected (at hour "24" after first aspercetin dose) with 10^6 UFC of *B. abortus* 2308. Mice were sacrificed seven days post-infection. Bars represent the error standard. The *p*-value <0,05 (*) is indicated.

Data curation, Funding acquisition, Methodology, Resources, Validation. **Alexandra Rucavado:** Validation, Conceptualization, Resources, Methodology, Funding acquisition. **Elías Barquero-Calvo:** Conceptualization, Data curation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the National Academy of Science of Costa Rica and the Vice-rectory for Research of the University of Costa Rica (FEES-CONARE: 741-B4-660, 741-B8-134, 803-C2-651 and 803-C0-456).

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