**Brucella abortus**–infected platelets modulate the activation of neutrophils

Aldana Trotta1, M Ayelén Milillo1, Agustina Serafino1, Luis A Castillo1, Federico Birnberg Weiss1, M Victoria Delpino2, Guillermo H Giambartolomei2, Gabriela C Fernández1 & Paula Barrionuevo1

1 Instituto de Medicina Experimental (CONICET-Academia Nacional de Medicina), Buenos Aires, Argentina
2 Instituto de Inmunología, Genética y Metabolismo (INIGEM), CONICET, Buenos Aires, Argentina

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**Correspondence**

Paula Barrionuevo and Aldana Trotta, Instituto de Medicina Experimental (CONICET-Academia Nacional de Medicina), Pacheco de Melo 3081, C1425AUM Buenos Aires, Argentina. E-mail: pbarrion2004@yahoo.com.ar and aldanatrotta@hotmail.com

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**Abstract**

Brucellosis is a contagious disease caused by bacteria of the genus *Brucella*. Platelets (PLTs) have been widely involved in the modulation of the immune response. We have previously reported the modulation of *Brucella abortus*–mediated infection of monocytes. As a result, PLTs cooperate with monocytes and increase their inflammatory capacity, promoting the resolution of the infection. Extending these results, in this study we demonstrate that patients with brucellosis present slightly elevated levels of complexes between PLTs and both monocytes and neutrophils. We then assessed whether PLTs were capable of modulating functional aspects of neutrophils. The presence of PLTs throughout neutrophil infection increased the production of interleukin-8, CD11b surface expression and reactive oxygen species formation, whereas it decreased the expression of CD62L, indicating an activated status of these cells. We next analyzed whether this modulation was mediated by released factors. To discriminate between these options, neutrophils were treated with supernatants collected from *B. abortus*–infected PLTs. Our results show that CD11b expression was induced by soluble factors of PLTs but direct contact between cell populations was needed to enhance the respiratory burst. Additionally, *B. abortus*–infected PLTs recruit polymorphonuclear (PMN) cells to the site of infection. Finally, the presence of PLTs did not modify the initial invasion of PMN cells by *B. abortus* but improved the control of the infection at extended times. Altogether, our results demonstrate that PLTs interact with neutrophils and promote a proinflammatory phenotype which could also contribute to the resolution of the infection.

**INTRODUCTION**

Brucellosis is a contagious disease caused by bacteria of the *Brucella* genus, which can affect cattle and humans. In most cases, clinical diagnosis of human brucellosis is problematic owing to its wide spectrum of both local and systemic manifestations. Among other diverse hematological alterations, patients with brucellosis frequently present with a reduction in the number of circulating platelets (PLTs; i.e. thrombocytopenia). Nevertheless, the pathophysiology of this manifestation remains unknown.

PLTs are small, enucleated and specialized cells derived from megakaryocytes which circulate in the bloodstream. Although they have been broadly described as being responsible for hemostatic balance and vascular integrity, over the last decade evidence has highlighted a central role of PLTs in the immune response regulation. PLTs have an extensive variety of receptors for both immunological and pathogenic molecules which may resemble those found on professional phagocytes. Upon activation of these receptors, PLTs are quickly activated and degranulated. Many of the immunological functions of PLTs are derived from the array of adhesion molecules and soluble mediators present within their granules, which might drive the consequent interaction with numerous leukocyte populations, especially with...
monocytes and neutrophils.\textsuperscript{13,16–20} We have previously described the collaboration between PLTs and monocytes during \textit{Brucella abortus}–mediated infection.\textsuperscript{21} Our results demonstrate that PLTs improve the invasion of monocytes and macrophages by \textit{B. abortus} and consequently form complexes with infected monocytes/macrophages. Furthermore, the presence of PLTs enhances the proinflammatory phenotype of infected monocytes, increasing the secretion of inflammatory cytokines and chemokines, and the expression of CD40 and CD54 (ICAM-1). This enhancement of the proinflammatory capacity of monocytes improves the control of \textit{B. abortus} inside the infected monocytes, which could promote resolution of the infection.\textsuperscript{21}

Polymorphonuclear (PMN) cells usually constitute the first line of defense against bacterial infections.\textsuperscript{22} Nevertheless, the specific role of neutrophils in \textit{B. abortus}–mediated infection remains controversial. Several studies performed \textit{in vivo} reveal that \textit{B. abortus} does not induce a significant recruitment of neutrophils neither to the site of infection nor to the spleen during the first \textit{48 h} of infection.\textsuperscript{23,24} At the same time, some studies performed in different models of neutropic mice demonstrate that PMN cells do not play a significant role in early stages of \textit{B. abortus}–mediated infection.\textsuperscript{23,25} In line with these results, several authors have revealed that the PMN cells might act as a “trojan horse” for \textit{Brucella}, carrying the bacteria toward monocytes, lymph nodes and the reticular endothelial system.\textsuperscript{26–29} Otherwise, it has been shown that depletion of neutrophils allows the efficient elimination of \textit{B. abortus} at late stages of the disease, which indicates an inhibitory effect of PMN cells on the adaptive immune response.\textsuperscript{35} \textit{In vitro} studies have also presented controversial results. Although \textit{B. abortus} can be internalized by PMN cells,\textsuperscript{30,31} it inhibits reactive oxygen species (ROS) formation and killing actions of PMN cells, surviving within PMN cells for extended periods.\textsuperscript{23,28,32} By contrast, we have previously demonstrated that heat-killed \textit{B. abortus} and its lipoproteins are able to activate neutrophils, inducing the expression of CD35 and CD11b while decreasing the expression of CD62L.\textsuperscript{33} Moreover, \textit{B. abortus} lipoproteins primed neutrophils for ROS production as well as promoted neutrophil migration and survival.\textsuperscript{35} However, all these \textit{in vitro} studies have only been performed with isolated PMN cells, which excludes the possibility of understanding the involvement of other cell populations present at the site of infection.

During the infection, \textit{B. abortus} present extracellular dissemination phases in order to reach their preferential niche, the macrophage. During these phases, PLTs and both monocytes and neutrophils are able to encounter bacteria and internalize them.\textsuperscript{2,3,34} Therefore, we focus our study on the role of PLTs in the modulation of neutrophils immune response against \textit{B. abortus}. We particularly investigated whether PLTs interact with neutrophils and/or modulate their activation during \textit{B. abortus} infection.

**RESULTS**

**Patients with brucellosis present slightly elevated levels of complexes**

We have previously demonstrated an increase in the establishment of monocyte–PLT complexes during \textit{B. abortus} infection.\textsuperscript{21} However, these experiments had only been performed \textit{in vitro}. Thus, we examined whether this increase in circulating complexes also occurs in \textit{in vivo} \textit{Brucella} infection. Consequently, the presence of monocyte–PLT complexes was evaluated in patients with chronic brucellosis. For this, whole blood was stained with anti-CD61 and anti-CD14 antibodies and the presence of monocyte–PLT complexes (CD14\textsuperscript{+}CD61\textsuperscript{+}) was assessed within the CD14\textsuperscript{+} gate by flow cytometry (Figure 1a). As shown in Figure 1b, patients with brucellosis indeed presented an increase in the number of circulating monocyte–PLT complexes. Next, we evaluated whether the ability of PLTs to establish complexes was restricted to monocytes or it could be extended to other immune cells such as lymphocytes and/or neutrophils. To evaluate the presence of lymphocyte–PLT complexes, whole blood was stained with anti-CD3 or anti-CD19, and anti-CD61 antibodies. Then, the presence of T-lymphocyte–PLT (CD3\textsuperscript{+}CD61\textsuperscript{+}) or B-lymphocyte–PLT complexes (CD19\textsuperscript{+}CD61\textsuperscript{+}) within the lymphocytes gate was assessed by flow cytometry. Only a small percentage of lymphocytes was associated with PLTs in blood from healthy donors and patients with brucellosis and no significant differences were found (Supplementary figure 1). Finally, we evaluated the presence of complexes between PMN cells and PLTs. Once more, whole blood was stained as described in Figure 1a and the presence of PMN cell–PLT complexes (CD14\textsuperscript{+}CD61\textsuperscript{+}) within the PMN cell gate was assessed by flow cytometry. Only a small percentage of lymphocytes was associated with PLTs in blood from healthy donors and patients with brucellosis and no significant differences were found (Supplementary figure 1). Overall, these results demonstrate that patients with brucellosis present slightly elevated levels of complexes between PLTs and both monocytes and neutrophils.

**\textit{Brucella abortus} promotes formation of PLT–PMN cell complexes**

The presence of circulating PLT–PMN cell complexes in patients with brucellosis led us to focus on the capability
of PLTs to interact and/or modulate the activation of these cells. To start with, we modeled the formation of PLT–PMN cell complexes observed in patients with brucellosis by infecting whole blood in vitro. For this, whole blood was infected with *B. abortus* and stained with anti-CD61 and anti-CD14 antibodies. Then, the presence

**Figure 1.** Patients with brucellosis present slightly elevated levels of PLT–PMN cell and PLT–monocyte complexes. Flow cytometry analysis of whole blood from healthy donors or patients with diagnosed brucellosis. Blood samples were stained with anti-CD61PE and anti-CD14PerCP antibodies and analyzed by flow cytometry. (a) Gating strategy to identify complexes between PLTs and monocytes. (b) Quantification of monocyte–PLT complexes (CD14+CD61+). (c) Gating strategy to identify complexes between PLTs and PMN cell. (d) Quantification of PMN–PLT complexes (CD14+CD61 within the PMN cell gate). Data are expressed as the percentage of leukocytes associated with PLTs ± s.e.m. of four independent experiments. In b and d, each dot represents data corresponding to an individual donor. *P < 0.05. FSC, forward scatter; PLTs, platelets; PMN, polymorphonuclear; SSC, side scatter.
of PLT–PMN cell complexes (CD14−CD61+) was assessed within the PMN cell gate by flow cytometry as described previously (Figure 2a, b). As shown in Figure 2a–c, B. abortus increased the percentage of neutrophils bound to PLTs (% of CD14−CD61+ cells), although the quantity of adhered PLTs per neutrophil (CD61 expression on cells from the CD14−CD61+ quadrant) was not modified (Figure 2d). Next, we focused on the kind of interaction between PLTs and neutrophils. To assess this, neutrophils were incubated with PLTs (PMN cells:PLTs ratio of 1:100), in the presence of B. abortus (multiplicity of infection 100 with respect to neutrophils) for 30 min. Then, neutrophils were stained with anti-CD11b (red) and PLTs with anti-CD61 (green) antibodies. Finally, samples were evaluated by confocal microscopy. We observed that PLTs directly bind to neutrophils when B. abortus was present (Figure 2e). Interestingly, we could also observe PLTs inside neutrophils (Figure 2e). Overall, these results reveal that the presence of B. abortus enhances the establishment of PLT–PMN cell complexes. Moreover, during B. abortus infection neutrophils not only directly bind PLTs but are also able to internalize them.

**PLTs promote B. abortus–mediated activation of PMN cells**

After studying the interaction between PLTs and neutrophils in the context of B. abortus infection, we decided to evaluate the ability of PLTs to modulate the activation of PMN cells. For this, PMN cells were infected with B. abortus with or without PLTs. Then, supernatants were collected for interleukin-8 (IL-8) quantification by enzyme-linked immunosorbent assay, and the expression of CD11b and CD62L (L-selectin) on PMN cells was measured by flow cytometry. As we have previously demonstrated,32 B. abortus infection increased the secretion of IL-8 and CD11b expression while it decreased CD62L expression (Figure 3a–c), a compatible response with neutrophil activation. The presence of PLTs during PMN cell infection enhanced the secretion of IL-8 and CD11b expression while it further decreased CD62L expression (Figure 3a–c). We then evaluated the respiratory burst associated with ROS generation, a PMN cell function related to bactericidal ability. As shown in Figure 3d, the presence of PLTs throughout PMN cell infection enhanced ROS production. Overall, these results demonstrate that PLTs enhance the activation and the microbicidal function of neutrophils in the context of B. abortus infection.

**PLT–PMN cell interaction is required for ROS production but not for CD11b modulation**

We have previously described the activation and degranulation of PLTs upon B. abortus recognition.21 We then studied whether the modulation of neutrophils functionality by PLTs during B. abortus infection was mediated by released factors and/or required physical contact between these cell populations. To answer this, PMN cells were stimulated with supernatants recollected from B. abortus–infected PLTs. PMN cells were also stimulated with N-formyl-methionyl-leucyl-phenylalanine or supernatants from bacteria or PLTs cultured alone as controls. Then, dimensions of PMN cells, CD11b expression and ROS formation were measured by flow cytometry. Culture supernatants from B. abortus–infected PLTs increase both the forward scatter of PMN cells (Figure 4a–c) and the surface expression of CD11b (Figure 4d), similar to formyl-methionyl-leucyl-phenylalanine treatment (Figure 4a–d). However, supernatants of B. abortus–infected PLTs did not modulate ROS production (Figure 4e). Overall, these results demonstrate the presence of soluble factors associated with the alteration of forward scatter and CD11b expression and that physical contact between PLTs and PMN cells might be dispensable for the modulation of these PMN cell activation parameters. However, physical interaction is necessary for modulation of the respiratory burst.

**Supernatant from infected PLTs promote PMN cell chemotaxis**

Taking into consideration that PLTs have been recently described as sentinel cells in several infections,35 we assessed whether PLTs are able to recruit neutrophils upon B. abortus infection. To evaluate the involvement of PLTs in this process, we designed two types of experiments using transwell plates. The first set of experiments was designed to determine the ability of the supernatants from B. abortus–infected PLTs to function as a chemotactic stimulus. For this, supernatant from B. abortus, PLTs or B. abortus–infected PLTs were placed in the bottom compartment of the transwell plate and PMN cells were seeded in the upper compartment. As shown in Figure 5a, supernatants recollected from B. abortus–infected PLTs could induce PMN cell chemotaxis compared with those collected from bacteria or PLTs alone. The other set of experiments was designed to evaluate the capacity of these supernatants to activate PMN cells, promoting the subsequent migration against another chemotactic stimulus. For this, PMN cells were pretreated with supernatants collected from B. abortus, PLTs or B. abortus–infected PLTs for 30 min. Afterwards, cells were seeded in the upper compartment of a transwell plate. In the lower compartment, pleural effusion from patients with tuberculosis was placed as a generic chemotactic stimulus. Finally, plates were
Figure 2. *Brucella abortus* (B.) promotes PLT–PMN cell complexes formation in whole blood. Flow cytometry analysis of whole blood (a) uninfected or (b) quantification of PLT–PMN cell complexes within the PMN cell gate. Data are expressed as the percentage of leukocytes associated with PLTs ± s.e.m. of three independent experiments. (c) Whole blood infected with B.a for 30 min. Blood samples were stained with anti-CD61PE and anti-CD14PerCP antibodies (Abs) and analyzed by flow cytometry to identify PLT–leukocyte complexes. (d) CD61 expression in PLT-bearing leukocytes (CD14+CD61+ within the PMN cell gate). Bars represent the arithmetic means ± s.e.m. of three experiments. (e) Confocal micrographs of PMN cells infected or not with B.a in the presence of PLTs for 30 min. Neutrophils were stained with an antihuman CD11b primary Ab followed by an antimouse IgG2aAlexa-546 secondary Ab (red). PLTs were stained with an antihuman CD61 primary Ab followed by an antimouse IgG1Alexa-488 secondary Ab (green). The micrographs are representative of three different experiments and the number of cells analyzed per experimental group was 200. White arrow indicates internalized PLTs. Blue arrows indicate PLTs bound to PMN cells while orange arrows indicate free PLTs. PMN cell:PTL:Ba 1:100:100. ***P < 0.001 versus uninfected. DIC, differential interference contrast; FSC, forward scatter; MFI, mean fluorescence intensity; NS, not significant; PLTs, platelets; PMN, polymorphonuclear; SSC, side scatter.
incubated for 3 h at 37°C. Then, media from the lower chamber was harvested and migrated cells were counted by flow cytometry. As shown in Figure 5b, PMN cell migration was increased when these cells were treated with supernatants from *B. abortus*–stimulated PLTs, but not when PMN cells were stimulated with supernatants from bacteria or PLTs alone. Overall, these results indicate that supernatants of *B. abortus*–infected PLTs act as both a chemotactic factor and a priming factor, promoting the migration of PMN cells to the site of infection.

**PLTs promote *B. abortus* control and elimination by neutrophils**

Finally, we studied whether the presence of PLTs affects the time course of *B. abortus* infection. Different microbicidal mechanisms have been described in which PMN cells can modify both the intracellular and extracellular bacterial load. We first focused on the intracellular mechanisms of bacteria elimination. For this, PMN cells were infected with *B. abortus* in the presence or absence of PLTs sat different ratios for 1 h. Then, extracellular bacteria were eliminated by addition of antibiotics and cells were incubated for 2 or 3 additional hours. PMN cells were then lysed, and the number of viable intracellular bacteria was determined by plating the lysates on tryptose soy agar. To discriminate between bacteria inside PLTs and inside PMN cells, we performed the colony-forming units (CFUs) count of PLTs alone concomitantly with the infection of PMN cells in the presence of PLTs. The number of bacteria recovered from PLTs were negligible and did not change over time. Nevertheless, the CFU count representative of bacteria inside PMN cells was obtained by extracting the possible contribution of PLTs to the total number of bacteria recovered in all treatments. As shown in Figure 6a, the presence of PLTs did not modify the initial invasion of PMN cells by *B. abortus*. However, the presence of PLTs improved the control of infection at extended times (Figure 6a). This result demonstrates that PLTs promote PMN cell activation and respiratory burst, enhancing the control and elimination of *B. abortus* within infected PMN cells.

We next evaluated whether PLTs are also able to modulate extracellular killing mechanisms. For this, isolated neutrophils were infected with *B. abortus* in the presence of different concentrations of PLTs for 1, 2 or 3 h in a standard medium without antibiotics. After incubation, PMN cells were separated by centrifugation and free cells supernatants were plated to determine the extracellular CFU count. As shown in Figure 6b, PMN cells partly decreased the number of extracellular *B. abortus*. Moreover, the presence of PLTs only increased the bacterial elimination at 1 h postinfection (Figure 6b). For both intracellular and extracellular elimination, the
effect of PLTs on the CFU count is not because of loss of PMN cell viability (Supplementary figure 2). In summary, these results suggest that PLTs first enhance the extracellular elimination of the bacteria but then increase the PMN cell activation and promote the elimination of the phagocyted bacteria.

DISCUSSION
To the best of our knowledge, this is the first study exploring the role of PLTs in the response of PMN cells against *B. abortus* infection. Particularly, here we demonstrate that *B. abortus* infection rises the number of PLT–PMN cell complexes. Moreover, PLTs enhance the *B. abortus*–mediated activation of PMN cells, thus improving the restraint and elimination of *B. abortus* within infected PMN cells.

Extending our previously published results, we demonstrate that patients with brucellosis present elevated levels of PLT–monocyte complexes. Interestingly, we also observe a slightly increased number of circulating PLT–PMN cell complexes in these patients. It has been previously described that healthy donors have a low percentage of PMN cells associated with PLTs in circulation. However, and in agreement with our results, it has been reported that this percentage is increased in several pathologies from diverse etiology such as infections, sterile inflammation, autoimmunity and cancer metastasis.
It has been proved that the activation of PLTs and the establishment of complexes between PLTs and leukocytes contribute to the decreased PLTs count observed during the infection with acute simian immunodeficiency virus in macaques. Thus, our recent findings might suggest the formation of PLT–monocyte and PLT–PMN cell complexes as one of the mechanisms involved in the thrombocytopenia frequently observed in patients with chronic brucellosis. Nevertheless, other mechanisms and factors involved in brucellosis-associated thrombocytopenia remain to be explored.

Figure 5. Supernatant from Brucella abortus–infected platelets (PLTs) promotes polymorphonuclear (PMN) cells transmigration. (a) Isolated PMN cells were seeded in the upper chamber of a transwell plate and supernatants collected from B. abortus, PLTs or B. abortus–infected PLTs were used as chemoattractant in the lower chamber. Results are expressed as percentage of migrated PMN cells relative to the seeded cells. (b) Isolated PMN cells were pretreated for 30 min with supernatants collected from B. abortus, PLTs or B. abortus–infected PLTs. Afterwards, cells were washed and seeded in the upper chamber of a transwell plate and pleural effusion from patients with tuberculosis were used as chemoattractant in the lower chamber. In both cases, the lower chamber media was harvested after 3-h incubation and the migrated cells were counted by flow cytometry. Results are expressed as percentage of migrated PMN cells relative to the seeded cells. Bars represent the mean ± s.d. of duplicates from a representative experiment of at least three performed with blood samples obtained from different donors. *P < 0.05; ***P < 0.001 versus medium. ###P < 0.001. NS, not significant; SN, supernatant.

Figure 6. Platelets (PLTs) promote Brucella abortus (Ba) restraint and elimination by neutrophils. (a) Isolated polymorphonuclear (PMN) cells were infected with Ba in the presence or absence of PLTs at different ratios for 1 h and then incubated with antibiotics for 2 or 3 additional hours. PMN cells were finally lysed and plated. (b) Isolated PMN cells were infected with Ba in the presence or absence of PLTs at different ratios for 1, 2 or 3 h. Free cell supernatants were then plated. Data are expressed as colony-forming units (CFUs) mL⁻¹ at each time point. Bars represent the mean ± s.d. of triplicates from a representative experiment of at least three performed with blood samples obtained from different donors. PMN cells:PTL:Ba 1:10:10 or 1:10:100 as specified. **P < 0.01; ***P < 0.001 versus PMN cells + Ba. ##P < 0.01; ###P < 0.001 versus Ba. PMN, polymorphonuclear.
We have previously demonstrated the establishment of PLT–monocyte complexes in *B. abortus*–mediated in vitro infection. Moreover, PLTs exhibited a particular disposition in these complexes, surrounding infected monocytes and creating rosettes. This finding led us to further study the distribution of PLTs within PLT–PMN cell complexes. Our confocal microscopy experiments showed that PLTs directly bind to neutrophils. Nevertheless, PLTs do not surround PMN cells creating rosettes. Surprisingly, this approach allowed us to find some internalized PLTs inside neutrophils. In our previous work, we proved that *B. abortus* is able to directly interact with and invade PLTs. Accordingly, we could hypothesize that infected PLT internalization by PMN cells could lead to an improved invasion of PMN cells by *B. abortus*. However, our results showed that the presence of PLTs did not modify the initial invasion of PMN cells by *B. abortus*. Instead, the presence of PLTs improved the extracellular restriction of the bacterial count at early times and the intracellular infection at extended times.

Here, we also demonstrate that PLTs enhance the activation of PMN cells in the context of *B. abortus* infection, as evidenced by the increase of IL-8 secretion, the upregulation of CD11b and the decrease of CD62L. Moreover, PLTs enhance the microbicidal function of PMN cells. In line with this, it has been proved that direct contact with PLTs enhance neutrophils activation, measured as CD11b upregulation. Moreover, *in vivo* studies have shown that establishment of PLT–PMN cell complexes, mainly mediated by P-selectin and P-selectin glycoprotein ligand-1, is a critical step for neutrophils recruitment to inflamed tissue. In addition, *in vitro* studies demonstrate that PLTs can prime neutrophils by upregulating integrins and adhesive response to chemokines. By contrast, PLTs are able to carpet the endothelium during inflammation, which may act as a bridge between PLT-primed neutrophils and the endothelial cells. P-selectin-mediated interaction has been identified as a key step for the mutual activation of leukocytes and endothelial cells. In agreement with these findings, our results showed an increase in the secretion of IL-8, suggesting a positive loop, which could promote the recruitment of circulating PMN cells and the adhesion to the activated endothelium in infected foci.

Even though both GPIIb/IIIa and P-selectin are frequently involved in the signaling pathways triggered in the PLT–leukocyte crosstalk, Li et al. have suggested that this crosstalk would be mainly dependent on soluble mediators, and not on cell–cell interactions. As an example, PLTs activating factor has been demonstrated to induce the priming of PMN cells and the upregulation of surface CD11b, whereas PLT factor 4 (CXCL4) has been proved to induce the activation of PMN cells upon its recognition by CD11b. Likewise, our conditioned media experiments indicated the involvement of soluble factors release by infected PLTs in the modulation of forward scatter and CD11b expression. Nonetheless, direct contact between PLTs and PMN cells is required for respiratory burst modulation. This result might be explained by considering that PLTs constitutively express the triggering receptor expressed on myeloid cells-1 (TREM-1) ligand, which has been proved to induce activation, phagocytosis and ROS production on PMN cells upon interaction with its receptor. More specifically, it has been shown that the ability of PLTs to enhance the lipopolysaccharide-induced respiratory burst is similar to the capacity of a TREM-1-specific agonist monoclonal antibody to induce ROS formation. In a previous study, Haselmayer et al. have proved that TREM-1 activation is necessary for the initiation of the respiratory burst. However, the formation of PLT–PMN cell complexes is stabilized by different interactions, because the establishment cannot be inhibited by blocking TREM-1 or TREM-1 ligand. Furthermore, these authors corroborate the importance of P-selectin and P-selectin glycoprotein ligand-1 interaction in the establishment of PMN cell–PLT complexes as previously described in the literature.

Following initial activation of PMN cells, the upregulation of integrins such as CD11b leads to interactions with endothelial cells. Moreover, this stable adhesion allows PMN cells to transmigrate through the endothelium. In this work, we have demonstrated that conditioned media collected from *B. abortus*–infected PLTs are able to recruit PMN cells in addition to prime them for further migration across the endothelium. In agreement with these results, it has been reported an absolute requirement of PLTs for neutrophils recruitment to inflamed tissue in some *in vivo* models such as allergy and nonallergic pulmonary inflammation. At the same time, *in vitro* results show that PLTs enhance both chemotaxis and chemokinesis of PMN cells, which depends on the presence of P-selectin in PLT supernatants. Several studies have described a diversity of molecules with chemotactic or chemokinetic activities on PMN cells such as β-thromboglobulin (CXCL7), PLT factor 4 (CXCL4), RANTES (CCL5) and leukotriene B4, among others.

Some of the results from studies performed *in vitro* with isolated PMN cells have been controversial regarding the restraint of *B. abortus*. Here, we have considered the presence of other cell populations relevant in the context of infections. In particular, we have demonstrated that the presence of PLTs increased the
microbicidal capacity of PMN cells, improving the restraint of the infection both within neutrophils and in the external media. Furthermore, our CFU experiments suggest a difference in the timeline of PLT modulation of PMN cell microbicidal activities because the extracellular mechanisms are enhanced within the first hour and the intracellular mechanisms appear to be increased later on.

Taking into account all of our results, we now have a broader vision of the role of PLTs in the context of \textit{B. abortus}–mediated infection. PLTs can directly interact with both monocytes and PMN cells, establishing complexes. These interactions promote the differentiation of these cell types into potent proinflammatory profiles with enhanced microbicidal capacity, contributing to the resolution of the infection. In this context, PLTs reduce the bacterial load in the bloodstream by promoting the uptake and restraint of \textit{B. abortus} by both monocytes and neutrophils. Altogether, our results suggest a protective role of PLTs in brucellosis and highlight the relevance of PLTs as contributors to host defense against \textit{Brucella}.

**METHODS**

**Ethics statement**

Human PMN cells and PLTs were exclusively purified from blood of healthy adult donors. As required by the IMEX Institute Ethical Committee, informed consent was provided by all blood donors prior to study participation.

**Bacteria**

\textit{Brucella abortus} strain S2308 was cultured in tryptose soy agar complemented with yeast extract (Merck Millipore, Burlington, MA, USA). Bacteria quantification was performed on stationary stage cultures by evaluation of the optical density at 600 nm. All experiments involving live \textit{Brucella} were performed in biosafety level 3 facilities, located at the INBIRS (Instituto de Investigaciones Biomédicas en Retrovirus y SIDA, Buenos Aires, Argentina) or at the ANLIS-Malbrán (Administración Nacional de Laboratorios e Institutos de Salud, “Dr Carlos G. Malbrán”, Buenos Aires, Argentina).

**Blood samples**

Blood samples were collected from healthy donors or chronic brucellosis patients who volunteered to participate in this study. In all cases, patients presented with a positive \textit{Rose Bengal assay} and serological test such as \textit{fixation of complement} and a high titer of \textit{Brucella}-specific antibodies measured by \textit{buffered plate antigen}. In line with the phases described in the literature, we consider a patient to be in the chronic phase after 12 months from the infection date and/or diagnosis.\textsuperscript{67,68} In the case of healthy donors, the group was selected to match the age range of the brucellosis patient group and with no history of chronic pathologies. All donors, both healthy and brucellosis patients, declared not having taken any medication for at least 10 days prior to the sampling. Blood was obtained by forearm venipuncture and collected directly into plastic tubes with sodium citrate 3.8\% (10:1; Merck Millipore). Informed consent was provided by all blood donors prior to study participation.

**Neutrophils (PMN cells) isolation**

PMN cells were isolated by centrifugation in a Ficoll-Hypaque gradient (GE Healthcare Life Sciences, Marlborough, MA, USA) followed by dextran sedimentation, as previously described.\textsuperscript{69} Erythrocytes were lysed by hypotonic shock and cells were washed. Isolated PMN cells were then resuspended in Roswell Park Memorial Institute-1640 medium complemented with 2\% heat-inactivated fetal calf serum (Gibco Invitrogen, Carlsbad, CA, USA) and used immediately. After the separation, May–Grünwald–Giemsa staining (CytoPrep) was performed in order to determine the purity of the isolation. In all cases, the experiments were performed with PMN cells with a purity of at least 96\%. Cell viability, quantified by trypan blue exclusion test, was more than 95\% in all experiments.

**Platelets**

PLT-rich plasma was isolated by centrifugation of the blood samples. To avoid contamination with leukocytes, only the upper 75\% of the plasma was collected. PLT-rich plasma was then treated with 75 nm of prostaglandin I\(_2\) (Cayman Chemical, Ellsworth, MI, USA) and centrifuged. Washed PLTs were then resuspended in media (Roswell Park Memorial Institute-1640 medium).

**In vitro infections**

PMN cells (\(0.5 \times 10^6 \text{ mL}^{-1}\)) were infected with \textit{B. abortus} (strain S2308) in the absence or presence of PLTs (PMN cells : \textit{B. abortus} : PLTs 1 : 100 : 100). All infections were done in standard medium without any antibiotics for 30 min. In all cases, uninternalized bacteria were removed by washing and cells were maintained in culture medium supplemented with gentamicin (100 \(\mu\text{g mL}^{-1}\)) and streptomycin (50 \(\mu\text{g mL}^{-1}\)). All incubations were performed in 5\% CO\(_2\) atmosphere at 37\°C.

In one set of experiments, supernatants were harvested, filtered and stored at \(-70\°C\) for later quantification by enzyme-linked immunosorbent assay. In a parallel set of experiments, cells were identically infected and then assessed by flow cytometry.

**Leukocyte–PLT complexes quantification**

The whole blood from healthy donors was infected \textit{in vitro} with \textit{B. abortus} or left untreated for 30 min at 37\°C. In an
additional set of experiments, blood was obtained from healthy donors or patients with brucellosis. In all cases, blood samples were stained with antihuman CD61PE (BD Biosciences, San Jose, CA, USA) and antihuman CD14PerCP (BioLegend, San Diego, CA, USA), anti-CD3FITC (BD Biosciences) or anti-CD19PerCP (BD Biosciences) antibodies and fixed. Finally, red blood cells were eliminated with a commercial Lysing Solution (BD FACS; BD Biosciences) and assessed by flow cytometry as described in each figure. Data were analyzed using FlowJo version 7.6 LLC (Ashland, OR, USA).

**PLT supernatants for PMN cell stimulation**

PLTs (1 × 10^7 mL⁻¹) were infected with *B. abortus* (PLTs:B. abortus ratio of 1:1) for 24 h. Then, supernatants were harvested, filtered and stored at −70°C until use. As controls, supernatants from PLTs or *B. abortus* incubated alone for 24 h were used.

**CFU quantification**

**Intracellular CFU count**

Isolated neutrophils were infected with *B. abortus* (multiplicity of infection 10) in the presence of different concentrations of PLTs (PMN cells:B. abortus:PLTs 1:10:10 and 1:10:100) for 1, 2 or 3 h in regular medium without any antibiotics. Then, PMN cells were washed and treated with gentamicin (100 µg mL⁻¹) and streptomycin (50 µg mL⁻¹) for 1, 2 or 3 additional hours as specified. Finally, PMN cells were lysed with Triton X-100 (0.01% v/v) and then plated in treptose soy agar complemented with yeast extract. After 4 days, the CFUs were analyzed.

As a control, the CFU count of PLTs alone incubated with *B. abortus* was performed concomitantly with the PMN cell infection in the presence of PLT. The number of microorganisms recovered from PLTs were negligible and do not change over time. Nevertheless, this contribution was excluded from the final CFU count.

**Extracellular CFU count**

Isolated neutrophils were incubated with *B. abortus* (multiplicity of infection 10) in the presence of different concentrations of PLTs (PMN cells:B. abortus:PLTs 1:10:10 and 1:10:100) for 1, 2 or 3 h in regular medium without antibiotics. After incubation, PMN cells were separated by centrifugation. The supernatants of free cells were then plated in treptose soy agar complemented with yeast extract. CFUs were analyzed 4 days after plating.

**Enzyme-linked immunosorbent assay**

Human IL-8 (BioLegend) concentration was measured by the enzyme-linked immunosorbent assay, using matching cytokine-specific antibodies as instructed by the manufacturer.

**Flow cytometry**

Isolated neutrophils (0.5 × 10^6 mL⁻¹) were infected in the presence or absence of PLTs for 30 min. Then, cells were washed and stained with antihuman CD11bPE antibody (BioLegend) or antihuman CD62L FITC (ImmuNoTools, Friesoythe, Lower Saxony, Germany) and assessed by flow cytometry. Data were analyzed using FlowJo version 7.6 (LLC).

**ROS generation**

ROS production was assessed using a derivat of rhodamine named Dihydrorhodamine 123, as described by Leech et al. In brief, isolated neutrophils were incubated with 1 µM of Dihydrorhodamine 123 for 15 min at 37°C. Then, cells were incubated with PLTs and/or *B. abortus* for an additional 30 min. Immediately after this step, the green fluorescence was assessed by flow cytometry.

**Chemotaxis assay**

A suspension of isolated PMN cells (4 × 10^5 cells in 75 µL) in Roswell Park Memorial Institute-1640 medium supplemented with 2% fetal bovine serum was seeded in the upper chamber of a transwell plate (polycarbonate membrane with 5-µm pore; Corning, Corning, NY, USA); 230 µL of supernatant collected from *B. abortus*, PLTs or *B. abortus*–infected PLTs was used in the lower chamber as chemoattractant. Plates were incubated for 3 h at 37°C under a 5% CO₂ atmosphere. Then, the media in the lower chamber was harvested and migrated cells were counted by flow cytometry. Results are expressed as the percentage of migrated cells relative to the seeded cells.

**Modulation of chemotaxis assay**

Isolated PMN cells were treated with supernatants collected from *B. abortus*, PLTs or *B. abortus*–infected PLTs at 37°C for 30 min. Cells were then washed and resuspended in Roswell Park Memorial Institute-1640 medium supplemented with 2% fetal bovine serum. Pretreated PMN cells (4 × 10^5 cells in 75 µL) were then seeded in the upper chamber of a transwell plate (polycarbonate membrane with 5-µm pore; Corning) and pleural effusion from patients with tuberculosis were used as chemoattractant in the lower chamber. As described previously, the lower chamber media was harvested after 3-h incubation and the migrated cells were counted by flow cytometry.

**Confocal microscopy**

Isolated neutrophils (2.5 × 10^5 neutrophils/well) were seeded onto coverslips coated with 7.5 ng mL⁻¹ of poly L-lysine in a
24-well plate. Then, PMN cells were infected with B. abortus in the presence or absence of PLTs for 1 h. After this step, cells were fixed with 2% paraformaldehyde for 20 min. Then, PLTs were stained with an anti-human CD61FITC primary antibody (Clone VI-PL2; BD Bioscience) tracked by an antinmouse IgG1Alexa 488 secondary antibody (Molecular Probes Life Technologies, Eugene, OR, USA). By contrast, PMN cells were stained with an antihuman CD11bPE primary antibody (Clone D12; BD Bioscience) tracked by an antimouse IgG2aAlexa 546 secondary antibody (Molecular Probes Life Technologies). Finally, slides were mounted with Aqua-Poly/ Mount (Polysciences, Hirschberg an der Bergstrasse, Germany) and evaluated using an FV-1000 confocal microscope with Plan Apochromatic 60× NA1.42 objective (Olympus, Shinjuku, Tokyo, Japan). Our confocal microscope operates with the multiline Ar laser (458, 488 and 515 nm) and HeNe (G) laser (543 nm) complemented with the emission filters BA 505–525 nm and BP 560–600 nm. The acquired images were analyzed with FIJI software, Open Source (Madison, WI, USA).

Statistical analysis

Results were evaluated with one- or two-way ANOVA followed by Tukey’s test. These analyses were performed using GraphPad Prism software (La Jolla, CA, USA).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Aldana Trotta: Conceptualization; Data curation; Investigation; Writing-original draft. M Ayelin Milillo: Investigation. Agustina Serafino: Investigation. Luis A Castillo: Investigation. Federico Birnberg Weiss: Investigation. M Victoria Delpino: Investigation. Guillermo H Giambartolomei: Funding acquisition; Investigation; Writing-review & editing. Gabriela C Fernández: Investigation; Writing-review & editing. Paula Barrionuevo: Conceptualization; Funding acquisition; Investigation; Project administration; Supervision; Writing-original draft; Writing-review & editing.

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