DR M. VICTORIA DELPINO (Orcid ID : 0000-0003-2077-8509)

Article type : Original Article

# *Brucella abortus*-infected platelets modulate activation of neutrophils

## AUTHOR QUERY: please approve title change at proofs

**Running title:** Platelets modulate infected-PMN's functions

Aldana Trotta,[*,1] M. Ayelén Milillo,[*] Agustina Serafino,[*] Luis A. Castillo,[*] Federico Birnberg Weiss,[*] M. Victoria Delpino,[†] Guillermo H. Giambartolomei,[†] Gabriela C. Fernández,[*] and Paula Barrionuevo[*,1]

[*]Instituto de Medicina Experimental (CONICET-Academia Nacional de Medicina). Buenos Aires. Argentina. [†]Instituto de Inmunología, Genética y Metabolismo (INIGEM). CONICET. Buenos Aires. Argentina.

[1]**Corresponding author information:** Dr. Paula Barrionuevo and Lic. Aldana Trotta. Instituto de Medicina Experimental (CONICET-Academia Nacional de Medicina). Pacheco de Melo 3081. C1425AUM. Buenos Aires. Argentina. Phone: 54-11-4805-5759. Fax: 54-11-4805-0712. E-mail: pbarrion2004@yahoo.com.ar; aldanatrotta@hotmail.com

**Key words:** platelets, polymorphonuclear cells, *Brucella abortus*, immune-complexes, early infection, brucellosis

**ABSTRACT**

Brucellosis is a contagious disease caused by bacteria of the genus *Brucella*. Platelets 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, platelets 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 platelets and both monocytes and neutrophils. We then assessed whether platelets are capable of modulating functional aspects of neutrophils. The presence of platelets throughout neutrophils infection increased the production of IL-8, CD11b surface expression and ROS formation while 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 platelets. Our results show that CD11b expression was induced by platelet's soluble factors but direct contact between cell populations was needed to enhance the respiratory burst. Alternatively, *B. abortus*-infected platelets recruit PMN to the site of infection. Finally, the presence of platelets did not modify the initial invasion of PMN by *B. abortus* but improved the restraint of the infection at extended times. Altogether, our results demonstrate that platelets interact with neutrophils and promote a pro-inflammatory phenotype which could also contribute to the restraint of the infection.

## INTRODUCTION

Brucellosis is a contagious disease caused by bacteria of *Brucella* genus, which can affect cattle and humans[1]. Clinical diagnosis of human brucellosis commonly results problematic due to its wide spectrum of both local and systemic manifestations[1–3]. Among other diverse hematological alterations[4–8], patients with brucellosis frequently present a reduction in the number of circulating platelets (i.e. thrombocytopenia). Nevertheless, the pathophysiology of this manifestation remains unknown.

Platelets (PLT) are small, enucleated, and specialized cells derived from megakaryocytes which circulate in the blood stream[9]. Although they have been broadly described as responsible for hemostatic balance and vascular integrity[10], over the last decade evidence has highlighted a central role of platelets in the immune response regulation[10–12]. Platelets have an extensive variety of receptors for both immunological and pathogenic molecules which may resemble those found on professional phagocytes[13]. Upon activation of these receptors, platelets are quickly activated and degranulate[13–15]. Many of the immunological functions of platelets derive from the array of adhesion molecules and soluble mediators present within its granules, which might drive the consequent interaction with numerous leukocyte populations, especially with monocytes and neutrophils[13,16–20]. We have previously described the collaboration between platelets and monocytes during *B. abortus*-mediated infection[21]. Our results demonstrate that platelets improve the invasion of monocytes and macrophages by *B. abortus* and consequently form complexes with infected monocytes/macrophages. Furthermore, the presence of platelets enhances the pro-inflammatory phenotype of infected monocytes increasing the secretion of inflammatory cytokines and chemokines, and the expression of CD40 and CD54 (ICAM-1). This enhancement of monocytes pro-inflammatory capacity improve the restraint of *B. abortus* inside the infected monocytes, which could promote the infection resolution[21].

Polymorphonuclear cells (PMN) usually constitute the first line of defense against bacterial infections[22]. Nevertheless, the specific role of neutrophils in *B. abortus*-mediated infection remains controversial. Several studies performed *in vivo* reveal that *B. abortus* does not induce a significant recruitment of neutrophils neither to the site of infection nor the spleen during the first 48 h of infection[23,24]. At the same time, some studies performed in different models of neutropenic mice demonstrate that PMN do not play a significant role in early stages of *B. abortus*-mediated infection[23,25]. In line with these results, Gutierrez-Gimenez *et al.* have revealed the PMN might act as a "trojan horse" for *Brucella*, carrying the bacteria towards monocytes, lymph nodes and the

reticular endothelial system[26–29]. Otherwise, it has been shown that neutrophils depletion allows the efficient elimination of *B. abortus* at late stages of the disease, which indicate an inhibitory effect of PMN on the adaptive immune response[25]. *In vitro* studies have also presented controversial results. Although *B. abortus* can be internalized by PMN[30,31], it inhibits Reactive Oxygen Species (ROS) formation and killing actions of PMN, surviving within PMN for extended periods[23,28,32]. On the other hand, we have previously demonstrated that heat-killed *B. abortus* and its lipoproteins are able to activate neutrophils, inducing the expression of CD35 and CD11b while decreasing the expression of CD62L[33]. Moreover, *B. abortus* lipoproteins primed neutrophils for ROS production as well as promoted neutrophil migration and survival[33]. However, all these *in vitro* studies have only been performed with isolated PMN, which excludes the possibility of understanding the involvement of other cell populations present at the site of infection.

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

## RESULTS

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

We have previously demonstrated an increase in the establishment of monocyte-platelet complexes during *B. abortus* (*Ba*) infection[21]. However, these experiments had only been performed *in vitro*. Thus, we examined whether this increase in circulating complexes also occurs *in vivo Brucella* infection. Consequently, the presence of monocyte-platelet 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-platelet complexes ($CD14^+CD61^+$) was assessed within the $CD14^+$ gate by flow cytometry (Figure 1a). As shown in Figure 1b, patients with brucellosis indeed presented an increase in the number of circulating monocyte-platelet complexes. Next, we evaluated whether the ability of platelets 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-platelet complexes, whole blood was stained with anti-CD3 or anti-CD19, and anti-CD61 antibodies. Then the presence of T lymphocyte-platelet ($CD3^+CD61^+$) or B lymphocyte-platelet complexes ($CD19^+CD61^+$) within the lymphocytes gate was assessed by flow cytometry. Only a small percentage of lymphocytes was associated with platelets in blood from healthy donors and brucellosis patients and no significant differences were found (Supplementary figure 1). We finally evaluate the presence of complexes between polymorphonuclear cells (PMN) and platelets. Once more, whole blood was stained as described for Figure 1a and the presence of PMN-platelet complexes ($CD14^-CD61^+$) within the PMN gate was assessed by flow cytometry (Figure 1c). Interestingly, patients with brucellosis showed slightly increased the number of circulating PMN-platelet complexes (% of $CD14^-CD61^+$ cells) (Figure 1d). Overall, these results demonstrate that patients with brucellosis present slightly elevated levels of complexes between platelets and both monocytes and neutrophils.

### *B. abortus* promotes platelet–PMN complexes formation

The presence of circulating platelet-PMN complexes in brucellosis patients led us to focus on the capability of platelets to interact and/or modulate the activation of these cells. To start with, we model the formation of platelet-PMN complexes observed in brucellosis patients by infecting whole blood *in vitro*. For this, whole blood was infected with *B. abortus* and stained with anti-CD61 and anti-CD14 antibodies. Afterward, the presence of platelet–PMN complexes ($CD14^-CD61^+$) was assessed within the PMN gate by flow cytometry as described before (Figure 2a, b). As shown in Figure 2a-c, *B. abortus* was able to increase the percentage of neutrophils bound to

platelets (% of $CD14^{-}CD61^{+}$ cells) although the quantity of adhered platelets 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 platelets and neutrophils. To assess this, neutrophils were incubated with platelets (PMN: PLT ratio of 1:100), in presence of *B. abortus* (MOI 100 respect to neutrophils) for 30 minutes. Then, neutrophils were stained with anti-CD11b (red) and platelets with anti-CD61 (green) antibodies. Finally, samples were evaluated by confocal microscopy. We observed that platelets directly bind to neutrophils when *B. abortus* was present (Figure 2e). Interestingly, we could also observe platelets inside neutrophils (Figure 2e). Overall, these results reveal that the presence of *B. abortus* enhances the establishment of platelet-PMN complexes. Moreover, during *B. abortus* infection neutrophils not only directly bind platelets but are also able to internalize them.

**Platelets promotes a *B. abortus*-mediated activation of PMN**

After studying the interaction between platelets and neutrophils in the context of *B. abortus* infection, we decided to evaluate the ability of platelets to modulate the activation of PMN. For this, PMN were infected with *B. abortus* with or without the presence or platelets. Then, supernatants were collected for IL-8 quantification by ELISA, and the expression of CD11b and CD62L (L-selectin) on PMN was measured by flow cytometry. As we have previously demonstrated[33], *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 platelets during PMN 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 function related to bactericidal ability. As shown in Figure 3d, the presence of platelets throughout PMN infection enhanced ROS production. Overall, these results demonstrate that platelets enhance the activation and the microbicidal function of neutrophils in the context of *B. abortus* infection.

**Platelet-PMN interaction is required for ROS production but not for CD11b modulation**

We have previously described the activation and degranulation of platelets upon *B. abortus* recognition[21]. We then studied whether the modulation of neutrophils functionality by platelets during *B. abortus* infection was mediated by released factors and/or required physical contact between these cell populations. To answer this, PMN were stimulated with supernatants

recollected from *B. abortus*-infected platelets. PMN were also stimulated with N-formyl-methionyl-leucyl-phenylalanine (fMLP) or supernatants from bacteria or platelets cultured alone as controls. Then, dimensions of PMN, CD11b expression and ROS formation were measured by flow cytometry. Culture supernatants from *B. abortus*-infected platelets increase both the Forward Scatter (FSC) of PMN (Figure 4a-c) and the surface expression of CD11b (Figure 4d), similar to fMLP treatment (Figure 4a-d). However, supernatants of *B. abortus*-infected platelets did not modulate ROS production (Figure 4e). Overall, these results demonstrate the presence of soluble factors associated with the alteration of FSC and CD11b expression and that physical contact between platelets and PMN might be dispensable for the modulation of these PMN activation parameters. However, physical interaction is necessary for modulation of the respiratory burst.

**Supernatant from infected platelets promote PMN chemotaxis**

Taking into consideration that platelets have been recently described out as sentinel cells in several infections[35], we assessed whether platelets are able to recruit neutrophils upon *B. abortus* infection. To evaluate the involvement of platelets 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 platelets to function as a chemotactic stimulus. For this, supernatant from *B. abortus*, platelets or *B. abortus*-infected platelets were placed in the bottom compartment of the transwell plate and PMN were seeded in the upper compartment. As shown in Figure 5a, supernatants recollected from *B. abortus*-infected platelets were able to induce PMN chemotaxis compared to those collected from bacteria or platelets alone. The other set of experiments was designed to evaluate the capacity of these supernatants to activate PMN, promoting the subsequent migration against another chemotactic stimulus. For this, PMN were pre-treated with supernatants collected from *B. abortus*, platelets or *B. abortus*-infected platelets for 30 minutes. Afterward, cells were seeded in the upper compartment of a transwell plate. In the lower compartment, pleural effusion from tuberculosis patients was placed as a generic chemotactic stimulus. Finally, plates were 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 migration was increased when these cells were treated with supernatants from *B. abortus*-stimulated platelets, but not when PMN were stimulated with supernatants from bacteria or platelets alone. Overall, these results indicate that supernatants of *B. abortus*-infected platelets

act both as chemotactic factor and priming factor, promoting the migration of PMN to the site of infection.

**Platelets promote *B. abortus* restraint and elimination by neutrophils**

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

We next evaluated whether platelets are also able to modulate extracellular killing mechanisms. For this, isolated neutrophils were infected with *B. abortus* in the presence of different concentrations of platelets for 1, 2 or 3 h in 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 were able to partly decrease the number of extracellular *B. abortus*. Moreover, the presence of platelets only increased the bacterial elimination at 1 h post-infection (Figure 6b). Both for intracellular and extracellular elimination, the effect of platelets in the CFU count is not due to loss of PMN cell viability (Supplementary figure 2). In summary, these results suggest that platelets firstly enhance the extracellular elimination of the bacteria but then increase the PMN activation and promote the elimination of the phagocyted bacteria.

## DISCUSSION

As far as our knowledge, this is the first study exploring the role of platelets in the PMN response against *B. abortus* infection. Particularly, here we demonstrate that *B. abortus* infection rises the number of platelet-PMN complexes. Moreover, platelets enhance the *B. abortus*-mediated activation of PMN improving the restraint and elimination of *B. abortus* within infected PMN.

Extending our previously published results[21], we demonstrate that patients with brucellosis present elevated levels of platelet-monocyte complexes. Interestingly, we also observe a slightly increased number of circulating platelet-PMN complexes in these patients. It has been previously described that healthy donors have a low percentage of PMN associated with platelets in circulation[36,37]. 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[38–40].

It has been proved that the activation of platelets and the establishment of complexes between platelets and leukocytes contribute to the decreased platelet count observed during the infection with acute simian immunodeficiency virus in macaques [41]. Thus, our recent findings might suggest the formation of platelet-monocyte and platelet-PMN 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 remained to be explored.

We have formerly demonstrated the establishment of platelet-monocyte complexes in *B. abortus*-mediated *in vitro* infection. Moreover, platelets exhibited a particular disposition in these complexes, surrounding infected monocytes and creating rosettes[21]. This finding led us to further study the distribution of platelets within platelet-PMN complexes. Our confocal microscopy experiments showed that platelets directly bind to neutrophils. Nevertheless, platelets do not surround PMN creating rosettes. Surprisingly, this approach allowed us to find some internalized platelets inside neutrophils. In our previous work, we proved that *B. abortus* is able to directly interact with and invade platelets[21] Accordingly, we could hypothesize that infected platelet internalization by PMN could lead to an improved invasion on PMN by *B. abortus*. However, our results showed that the presence of platelets did not modify the initial invasion of PMN by *B. abortus*. Instead, the presence of platelets improved the extracellular restriction of the bacterial count at early times and the intracellular infection at extended times.

Here, we also demonstrate that platelets enhance the activation of PMN in the context of *B. abortus* infection, as evidenced by the increase of IL-8 secretion, the up-regulation of CD11b and the decrease of CD62L. Moreover, platelets enhance the microbicidal function of PMN. In line with this, it has been proved that direct contact with platelets enhance neutrophils activation, measured as CD11b upregulation[42,43]. Moreover, *in vivo* studies have shown that establishment of platelet-PMN complexes, mainly mediated by P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1)[44–47], is a critical step for neutrophils recruitment to inflamed tissue. In addition, *in vitro* studies demonstrate that platelets can prime neutrophils by upregulating integrins and adhesive response to chemokines[48]. On the other hand, platelets are able to carpet the endothelium during inflammation, which may act as a bridge between platelet-primed neutrophils and the endothelial cells[49]. P-selectin-mediated interaction has been identified as a key step for the mutual activation of leukocytes and endothelial cells[40,50,51]. In agreement with these evidences, our results showed an increase in the secretion of IL-8, suggesting a positive loop, which could promote the recruitment of circulating PMN 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 platelet–leukocyte crosstalk, Li *et al.* have suggested that this crosstalk would be mainly dependent on soluble mediators, and not on cell–cell interactions[52]. As an example, Platelets activating factor (PAF) has been demonstrated to induce the priming of PMN and the upregulation of surface CD11b[53,54] while Platelet factor 4 (PF4 or CXCL4) has been proved to induce the activation of PMN upon its recognition by CD11b[55]. Likewise, our conditioned media experiments indicated the involvement of soluble factors release by infected platelets in the modulation of FSC and CD11b expression. Nonetheless, direct contact between platelets and PMN is required for respiratory burst modulation. This result might be explained by considering that platelets constitutively express the TREM-1 ligand, which has been proved to induce activation, phagocytosis and ROS production on PMN upon interaction with its receptor[56]. More specifically, it has been shown the ability of platelets to greatly enhance the LPS-induced respiratory burst, resembling the ROS formation achieved by a TREM-1-specific agonist mAb[57–59]. In this work, Haselmayer *et al.* have proved that TREM-1 activation is necessary for the initiation of the respiratory burst. However, the formation of platelet-PMN complexes is stabilized by different interactions, since the establishment cannot be inhibited by blocking TREM-1 or TREM-1 ligand[59]. Furthermore, these authors corroborate the importance of P-selectin and PSGL-1

interaction in the establishment of PMN-PLT complexes[59] as previously described in the literature[44–47].

Following initial activation of PMN, the upregulation of integrins such as CD11b lead to interactions with endothelial cells. Moreover, this stable adhesion allows PMN to transmigrate through the endothelium. In this work, we have demonstrated that conditioned media collected from *B. abortus*-infected platelets are able to recruit PMN in addition to prime them for further migration across the endothelium. In agreement with these results, it has been reported an absolute requirement of platelets for neutrophils recruitment to inflamed tissue in some *in vivo* models such as allergy and non-allergic pulmonary inflammation[60,61]. At the same time, *in vitro* results show that platelets enhance both chemotaxis and chemokinesis of PMN, dependently of P-selectin presence in platelets supernatants[62]. Several studies have described a diversity of molecules with chemoattractant activities on PMN such as β-trhomboglobulin (CXCL7), PF4 (CXCL4), RANTES (CCL5) and Leukotriene B4, among others[63–66].

Some of the results from studies performed *in vitro* with isolated PMN have been controversial regarding the restraint of *B. abortus*[23,28,30–33]. Here, we have considered the presence of other cell population relevant in the context of infections. In particular, we have demonstrated that the presence of platelets increased the microbicidal capacity of PMN, 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 platelets modulation of PMN microbicidal activities since 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 platelets in the context of *B. abortus*-mediated infection. Platelets can directly interact both with monocytes and PMN, establishing complexes. These interactions promote the differentiation of these cell types into potent pro-inflammatory profiles with enhanced microbicidal capacity, contributing to the resolution of the infection. In this context, platelets reduce the bacterial load in the bloodstream by promoting the uptake and restraint of *B. abortus* both by monocytes and neutrophils. Altogether, our results suggest a protective role of platelets in brucellosis and highlight the relevance of platelets as contributors to host defense against *Brucella*.

## METHODS

### Ethics Statement

Human polymorphonuclear cells and platelets 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 conducting the study.

### Bacteria

*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 *Brucella* were performed in biosafety level 3 facilities, located whether at the INBIRS (Instituto de Investigaciones Biomédicas en Retrovirus y SIDA) 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 volunteer to participate in this study. In all cases, patients presented positive *Rose Bengal Assay* and serological test such as *Fixation of Complement* (FC) and a high titer of *Brucella*-specific antibodies measured by *Buffered Plate Antigen* (BPA). 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[67,68]. In the case of healthy donors, the group was selected to match the age range of the brucellosis patient group and have no history of chronic pathologies. All donors, both healthy and brucellosis patients, declared not had taken any medication for at least 10 days prior 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 conducting the study.

### Neutrophils (PMN) Isolation

Polymorphonuclear cells were isolated by centrifugation in a Ficoll-Hypaque gradient (*Ficoll* Pharmacia; *Hypaque*, Winthrop Products) followed by dextran sedimentation, as previously described[69]. Erythrocytes were lysed by hypotonic shock and cells were washed. Isolated PMN were then resuspended in RPMI-1640 complemented with 2% heat-inactivated fetal calf serum

(FCS) (*Gibco Invitrogen,* Carlsbad, CA, USA) and used immediately. After the separation, a May Grünwald Giemsa-stain of Cyto-preps was performed in order to determine the purity of the isolation. In all cases, the experiments were performed with PMN with a purity of at least 96%. Cells Viability, quantified by trypan blue exclusion test, was more than 95% in all the experiments.

**Platelets**

Platelet-rich plasma (PRP) was isolated by centrifugation of the blood samples. To avoid contamination with leukocytes, only the upper 75% of the Plasma was collected. PRP was then treated with 75 nM of prostaglandin $I_2$ (*Cayman Chemical,* Ellsworth, MI, USA) and centrifuged. Washed platelets were then resuspended in media (RPMI-1640).

***In vitro* Infections**

PMN cells ($0.5 \times 10^6$ $mL^{-1}$) were infected with *B. abortus (strain* S2308) in absence or present of platelets (PMN: *Ba*: PLT 1:100:100). All infections were done in standard medium without any antibiotics for 30 minutes. In all cases, uninternalized bacteria was remove by washing and cells were maintained in culture medium supplemented with gentamicin (100 µg $mL^{-1}$) and streptomycin (50 µg $mL^{-1}$). 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 ELISA. In a parallel set, cells were identically infected and then assessed by flow cytometry.

**Leukocyte-Platelet Complexes Quantification**

The whole blood from healthy donors was infected *in vitro* with *B. abortus* or left untreated for 30 min at 37°C. In an additional set of experiments, blood was obtained from healthy donors or brucellosis patients. In all cases, blood samples were stained with anti-human CD61PE (*BD Biosciences*, San Jose, CA, USA) and anti-human 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 describe in each figure. Data were analyzed using the software FlowJo® 7.6 (*LLC*).

**Platelet supernatants for PMN stimulation**

Platelets (1 x $10^7$ $mL^{-1}$) were infected with *B. abortus* (PLT: *Ba* ratio of 1:1) for 24 h. Then, supernatants were harvested, filtered and stored at -70°C until use. As control, supernatants from platelets or *B. abortus* incubated alone for 24 h were used.

**Colony Forming Units (CFU) quantification**

*Intracellular CFU count*

Isolated neutrophils were infected with *B. abortus* (Multiplicity of Infection or MOI, 10) in the presence of different concentrations of platelets (PMN: *Ba*: PLT 1:10:10 and 1:10:100) for 1 h in regular medium without any antibiotics. Then, PMN 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 tryptose-soy agar complemented with yeast extract. After 4 days, the CFU were analyzed.

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

*Extracellular CFU count*

Isolated neutrophils were incubated with *B. abortus* (MOI 10) in the presence of different concentrations of platelets (PMN: *Ba*: PLT 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. Free cells supernatants were then plated in tryptose-soy agar complemented with yeast extract. CFU were analyzed 4 days post-plated.

**ELISA (Enzyme Linked ImmunoSorbent Assay)**

Human IL-8 (*BioLegend*) concentration was measured by ELISA, using matching cytokine-specific antibodies as instructed by the manufacturer.

**Flow cytometry**

Isolated neutrophils (0.5 x $10^6$ $mL^{-1}$) were infected in presences or absence of platelets for 30 minutes. Then, cells were washed and stained with anti-human CD11bPE antibody (*BioLegend*) or

anti-human CD62LFITC (*ImmunoTools*, Friesoythe, Lower Saxony, Germany) and assessed by flow cytometry. Data were analyzed using FlowJo® 7.6 (*LLC*).

**Reactive Oxygen Species (ROS) Generation**

ROS production was assessed using a derivate of Rhodamine named DHR-123, as described by Leech *et al.*[70]. Briefly, isolated neutrophils were incubated with 1 μM DHR-123 for 15 min at 37°C. Then, cells were incubated with platelets and/or *B. abortus* for additional 30 min. Immediately after, the green fluorescence was assessed by flow cytometry.

**Chemotaxis Assay**

A suspension of isolated PMN (4 x $10^5$ cells in 75 μl) in RPMI 2% FBS was seeded in the upper chamber of a transwell plate (polycarbonate membrane with 5 μm pore) (*Corning*, Corning, NY, USA). 230 μl of supernatants collected from *B. abortus*, platelets or *B. abortus*-infected platelets were used in the lower chamber as chemoattractant. Plates were incubated for 3 h at 37°C under a 5% $CO_2$ atmosphere. Then, the media in the lower chamber was harvested and migrated cells were count by flow cytometry. Results were expressed as percentage of migrated cells relative to the seeded cells.

**Modulation of Chemotaxis Assay**

Isolated PMN were treated with supernatants collected from *B. abortus*, platelets or *B. abortus*-infected platelets at 37°C for 30 minutes. Afterwards, cells were washed and resuspended in RPMI 2% FBS. Pre-treated PMN (4 x $10^5$ 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 tuberculosis patients were used as chemoattractant in the lower chamber. As described before, the lower chamber media was harvested after 3 h incubation and the migrated cells were count by flow cytometry.

**Confocal microscopy**

Isolated neutrophils (2.5 x $10^5$ neutrophils/well) were seeded onto coverslips coated with 7.5 ng $mL^{-1}$ of Poly L-lysine in 24-well plate. Then, PMN were infected with *B. abortus* in presence or absence of platelets for 1 h. Afterwards, cells were fixed with 2% paraformaldehyde for 20 minutes. Then, platelets were stained with an anti-human CD61FITC primary Ab (Clone VI-PL2;

*BD Bioscience*) tracked by an anti-mouse IgG1Alexa 488 secondary Ab (*Molecular Probes Life Technologies*, Eugene, OR, USA). On the other hand, PMN cells were stained with an anti-human CD11bPE primary Ab (Clone D12; *BD Bioscience*) tracked by an anti-mouse IgG2aAlexa 546 secondary Ab (*Molecular Probes Life Technologies*). Finally, slides were mounted with Aqua PolyMount (*Polysciences*) and evaluated using a FV-1000 confocal microscope with Plan Apochromatic 60X NA1.42 objective (*Olympus*, Shinjuku, Tokyo, Japan). Our confocal microscope operates with Multiline Ar laser (458 nm, 488 nm, 515 nm) and HeNe(G) laser (543 nm) complemented with the emission filters BA 505-525 nm, and BP 560-660 nm. The acquired images were analyzed with FIJI software (*open source*).

**Statistical analysis**

Results were evaluated with one or two-way ANOVA followed by Tukey's test. These analyses were performed by GraphPad Prism software.

**ACKNOWLEDGMENTS**

We thank Horacio Salomon and the staff of the INBIRS (UBA, Buenos Aires) as well as the staff of the ANLIS-Malbrán (Buenos Aires) for allowing us to use the Biosafety Level 3 Laboratory facilities. We thank Dr. Santiago Garro for kindly providing the blood samples from chronic brucellosis patients. We also thank the technical assistance with confocal microscopies provided by Federico Fuentes. This work was supported by PICT 2016-0356, 2016-1945, and 2017-1393 grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT-Argentina), and by grant from Fundación Alberto J. Roemmers (2018-2020) (Argentina).

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

## REFERENCES


1. Young EJ. Human brucellosis. *Rev Infect Dis* 1983; **5**, :821–842.
2. Moral M. Enfermedades infecciosas: Brucelosis. Vol. **12**, Guía para el equipo de salud. Ministerio de Salud de la Nación Argentina; 2013.
3. Baldi PC, Giambartolomei GH. Immunopathology of *Brucella* infection. *Recent Pat Antiinfect Drug Discov* 2013; **8**, 18–26.
4. Sevinc A, Buyukberber N, Camci C, Buyukberber S, Karsligil T. Thrombocytopenia in brucellosis: case report and literature review. *J Natl Med Assoc* 2005; **97**, 290–293.
5. Ariza J, Corredoira J, Pallares R, *et al.* Characteristics of and risk factors for relapse of brucellosis in humans. *Clin Infect Dis* 1995; **20**, 1241–1249.
6. Franco MP, Mulder M, Gilman RH, Smits HL. Human brucellosis. *Lancet Infect Dis* 2007; **7**, 775–786.
7. Sari I, Altuntas F, Hacioglu S, *et al.* A multicenter retrospective study defining the clinical and hematological manifestations of brucellosis and pancytopenia in a large series: Hematological malignancies, the unusual cause of pancytopenia in patients with brucellosis. *Am J Hematol* 2008; **83**, 334–339.
8. Xie S, Zhou Y, Zheng R, *et al. Brucella*-induced thrombocytopenia: a retrospective study of 16 patients. *J Int Med Res* 2019; **47**, 3008–3013.
9. Clemetson KJ. Platelets and primary haemostasis. *Thromb Res* 2012; **129**, 220–224.
10. Kahn F, Hurley S, Shannon O. Platelets promote bacterial dissemination in a mouse model of streptococcal sepsis. *Microbes Infect* 2013; **15**, 669–676.
11. Gudbrandsdottir S, Hasselbalch HC, Nielsen CH. Activated platelets enhance IL-10 secretion and reduce TNF-alpha secretion by monocytes. *J Immunol* 2013; **191**, 4059–4067.
12. Wuescher LM, Takashima A, Worth RG. A novel conditional platelet depletion mouse model reveals the importance of platelets in protection against *Staphylococcus aureus* bacteremia. *J Thromb Haemost* 2015; **13**, 303–313.
13. Aslam R, Speck ER, Kim M, *et al.* Platelet Toll-like receptor expression modulates lipopolysaccharide-induced thrombocytopenia and tumor necrosis factor-alpha production *in vivo*. *Blood* 2006; **107**, 637–641.
14. Speth C, Loffler J, Krappmann S, Lass-Florl C, Rambach G. Platelets as immune cells in infectious diseases. *Futur Microbiol* 2013; **8**, 1431–1451.
15. Semple JW, Italiano Jr. JE, Freedman J. Platelets and the immune continuum. *Nat Rev Immunol* 2011; **11**, 264–274.
16. Cognasse F, Hamzeh H, Chavarin P, *et al.* Evidence of Toll-like receptor molecules on human platelets. *Immunol Cell Biol* 2005; **83**, 196–198.
17. Fitzgerald JR, Foster TJ, Cox D. The interaction of bacterial pathogens with platelets. *Nat Rev Microbiol* 2006; **4**, 445–457.
18. Ghasemzadeh M, Hosseini E. Platelet-leukocyte crosstalk: Linking proinflammatory responses to procoagulant state. *Thromb Res* 2013; **131**, 191–197.
19. Li C, Li J, Li Y, *et al.* Crosstalk between Platelets and the Immune System: Old Systems with New Discoveries. *Adv Hematol* 2012; **2012**, 384685.

20. von Bruhl ML, Stark K, Steinhart A, *et al.* Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice *in vivo*. *J Exp Med* 2012; **209**, 819–835.
21. Trotta A, Velasquez LN, Milillo MA, *et al.* Platelets Promote *Brucella abortus* Monocyte Invasion by Establishing Complexes With Monocytes. *Front Immunol* 2018; **9**, 1000.
22. McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P. Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell Host Microbe* 2012; **12**, 324–333.
23. Barquero-Calvo E, Chaves-Olarte E, Weiss DS, *et al. Brucella abortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection. *PLoS One* 2007; **2**, e631.
24. Conde-Álvarez R, Arce-Gorvel V, Iriarte M, *et al.* The lipopolysaccharide core of *Brucella abortus* acts as a shield against innate immunity recognition. *PLoS Pathog* 2012; **8(5)**, e1002675.
25. Barquero-Calvo. Neutrophil exert a supressive effect on TH1 responses to intracellular pathogen *Brucella abortus*. *PLoS One* 2013; **9(2)**, e1003167.
26. Gutiérrez-Jiménez C, Mora-Cartín R, Altamirano-Silva P, *et al.* Neutrophils as trojan horse vehicles for *Brucella abortus* macrophage infection. *Front Immunol* 2019; **10**, 1012.
27. Laskay T, Van Zandbergen G, Solbach W. Neutrophil granulocytes - Trojan horses for *Leishmania major* and other intracellular microbes? Vol. **11**, *Trends in Microbiology*. Elsevier Ltd; 2003.
28. Kreutzer DL, Dreyfus LA, Robertson DC. Interaction of polymorphonuclear leukocytes with smooth and rough strains of *Brucella abortus*. *Infect Immun* 1979; **23**, 737–742.
29. Canning PC, Roth JA, Tabatabai LB, Deyoe BL. Isolation of components of *Brucella abortus* responsible for inhibition of function in bovine neutrophils. *J Infect Dis* 1985; **152**, 913–921.
30. Braude AI. Studies in the pathology and pathogenesis of experimental brucellosis. *J Infect Dis* 1951; **89**, 87–94.
31. Ackermann MR, Cheville NF, Deyoe BL. Bovine Ileal Dome Lymphoepithelial Cells: Endocytosis and Transport of *Brucella abortus* Strain 19. *Vet Pathol* 1988; **25**, 28–35.
32. Martinez de Tejada G, Pizarro-Cerda J, Moreno E, Moriyon I. The outer membranes of *Brucella* spp. are resistant to bactericidal cationic peptides. *Infect Immun* 1995; **63**, 3054–3061.
33. Zwerdling A, Delpino M V, Pasquevich KA, *et al. Brucella abortus* activates human neutrophils. *Microbes Infect* 2009; **11**, 689–697.
34. Trotta A, Velásquez LN, Milillo MA, *et al.* Platelets Promote *Brucella abortus* Monocyte Invasion by Establishing Complexes With Monocytes. *Front Immunol* 2018; **9**, :1000.
35. Kim SJ, Jenne CN. Role of platelets in neutrophil extracellular trap (NET) production and tissue injury. Vol. **28**, *Seminars in Immunology*. Academic Press; 2016.
36. Zhang X, Majlessi L, Deriaud E, Leclerc C, Lo-Man R. Coactivation of Syk Kinase and MyD88 Adaptor Protein Pathways by Bacteria Promotes Regulatory Properties of Neutrophils. *Immunity* 2009; **31**, 761–771.
37. Silva MT. When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. *J Leukoc Biol* 2010; **87**, 93–106.
38. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. Vol. **11**, *Nature Reviews Immunology*. 2011.
39. Spörri R, Joller N, Hilbi H, Oxenius A. A Novel Role for Neutrophils As Critical Activators of NK Cells. *J*

*Immunol* 2008; **181**, 7121–7130.

40. Yang C-W, Strong BSI, Miller MJ, Unanue ER. Neutrophils Influence the Level of Antigen Presentation during the Immune Response to Protein Antigens in Adjuvants. *J Immunol* 2010; **185**, 2927–2934.
41. Metcalf Pate KA, Lyons CE, Dorsey JL, *et al.* Platelet activation and platelet-monocyte aggregate formation contribute to decreased platelet count during acute simian immunodeficiency virus infection in pig-tailed macaques. In: *Journal of Infectious Diseases* 2013. p. 874–883.
42. Stevens MG, Pugh GW, Tabatabai LB. Effects of gamma interferon and indomethacin in preventing *Brucella abortus* infections in mice. *Infect Immun* 1992; **60**, 4407–4409.
43. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients. *Cancer Res* 2001; **61**, 4756–4760.
44. Moore KL, Varki A, McEver RG. GMP-140 binds to a glycoprotein receptor on human neutrophils: Evidence for a lectin-like interaction. *J Cell Biol* 1991; **112**, 491–499.
45. Larsen E, Palabrica T, Sajer S, *et al.* PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by a lineage-specific carbohydrate, LNF III (CD15). *Cell* 1990; **63**, 467–474.
46. Hamburger SA, McEver RP. GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood* 1990; **75**, 550–554.
47. Moore KL, Patel KD, Bruehl RE, *et al.* P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *J Cell Biol* 1995; **128**, 661–671.
48. Page C, Pitchford S. Neutrophil and platelet complexes and their relevance to neutrophil recruitment and activation. *Int Immunopharmacol* 2013; **17**, 1176–1184.
49. Blomgran R, Desvignes L, Briken V, Ernst JD. *Mycobacterium tuberculosis* inhibits neutrophil apoptosis, leading to delayed activation of naive CD4 T cells. *Cell Host Microbe* 2012; **11**, 81–90.
50. Cassatella MA. The production of cytokines by polymorphonuclear neutrophils. *Immunol Today* 1995; **16**, 21–26.
51. Fernandes DM, Jiang X, Jung JH, Baldwin CL. Comparison of T cell cytokines in resistant and susceptible mice infected with virulent *Brucella abortus* strain 2308 . *FEMS Immunol Med Microbiol* 1996; **16**, 193–203.
52. Li N, Hu H, Lindqvist M, *et al.* Platelet-leukocyte cross talk in whole blood. *Arter Thromb Vasc Biol*. 2000; **20**, 2702–2708.
53. Zhu X, Tan Z, Chen J, Zhu M, Xu Y. Effects of ropivacaine on adhesion molecule CD11b expression and function in human neutrophils. *Int Immunopharmacol* 2010; **10**, 662–667.
54. Read RA, Moore EE, Moore FA, *et al.* Platelet-activating factor-induced polymorphonuclear neutrophil priming independent of CD11(B) adhesion. *Surgery* 1993; **114**, 308–313.
55. Lishko VK, Yakubenko VP, Ugarova TP, Podolnikova NP. Leukocyte integrin Mac-1 (CD11b/CD18, αMβ2, CR3) acts as a functional receptor for platelet factor 4. *J Biol Chem* 2018; **293**, 6869–6882.
56. Tateda K, Moore TA, Deng JC, *et al.* Early Recruitment of Neutrophils Determines Subsequent T1/T2 Host Responses in a Murine Model of *Legionella pneumophila* Pneumonia . *J Immunol* 2001; **166**, 3355–3361.
57. Fortin CF, Lesur O, Fulop T. Effects of TREM-1 activation in human neutrophils: Activation of signaling pathways, recruitment into lipid rafts and association with TLR4. *Int Immunol* 2007; **19**, 41–50.
58. Radsak MP, Salih HR, Rammensee H-G, Schild H. Triggering Receptor Expressed on Myeloid Cells-1 in

Neutrophil Inflammatory Responses: Differential Regulation of Activation and Survival. *J Immunol* 2004; **172**, 4956–4963.

59. Haselmayer P, Grosse-Hovest L, Von Landenberg P, Schild H, Radsak MP. TREM-1 ligand expression on platelets enhances neutrophil activation. *Blood* 2007; **110**, 1029–1035.
60. Kornerup KN, Salmon GP, Pitchford SC, Liu WL, Page CP. Circulating platelet-neutrophil complexes are important for subsequent neutrophil activation and migration. *J Appl Physiol* 2010; **109**, 758–767.
61. De Sanctis GT, Wolyniec WW, Green FHY, *et al.* Reduction of allergic airway responses in P-selectin-deficient mice. *J Appl Physiol* 1997; **83**, 681–687.
62. Bengtsson T, Frydén A, Zalavary S, *et al.* Platelets enhance neutrophil locomotion: Evidence for a role of P-selectin. *Scand J Clin Lab Invest* 1999; **59**, 439–449.
63. Brandt E, Petersen F, Ludwig A, *et al.* The beta-thromboglobulins and platelet factor 4: blood platelet-derived CXC chemokines with divergent roles in early neutrophil regulation. *J Leukoc Biol* 2000; **67**, 471–478.
64. Walz A, Dewald B, von Tscharner V, Baggiolini M. Effects of the neutrophil-activating peptide NAP-2, platelet basic protein, connective tissue-activating peptide III and platelet factor 4 on human neutrophils. *J Exp Med* 1989; **170**, 1745–1750.
65. Grommes J, Alard J-E, Drechsler M, *et al.* Disruption of platelet-derived chemokine heteromers prevents neutrophil extravasation in acute lung injury. *Am J Respir Crit Care Med* 2012; **185**, 628–636.
66. Palmantier R, Borgeat P. Thrombin-activated platelets promote leukotriene B4 synthesis in polymorphonuclear leucocytes stimulated by physiological agonists. *Br J Pharmacol* 1991; **103**, 1909–1916.
67. Pabuccuoglu O, Ecemis T, El S, *et al.* Evaluation of serological tests for diagnosis of brucellosis. *Jpn J Infect Dis* 2011; **64**, 272–276.
68. Young EJ. Clinical manifestations of human brucellosis. In: Young EJ, Corbel MJ, editors. Brucellosis Clinical and Laboratory Aspects *CRC Press*; 1989. p. 97.
69. Böyum A. Separation of leukocytes from blood and bone marrow. Introduction. *Scand J Clin Lab Investig Suppl* 1968; **97**, 7.
70. Leech M, Hutchinson P, Holdsworth SR, Morand EF. Endogenous glucocorticoids modulate neutrophil migration and synovial P- selectin but not neutrophil phagocytic or oxidative function in experimental arthritis. *Clin Exp Immunol* 1998; **112**, 383–388.

**FIGURE LEGENDS**

**Figure 1. Patients with brucellosis present slightly elevated levels of platelet-PMN and platelet-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 in order to identify complexes between platelets and monocytes. **(b)** Quantification of monocyte-platelet complexes ($CD14^{+}CD61^{+}$). **(c)** Gating strategy in order to identify complexes between platelets and PMN. **(d)** Quantification of PMN-platelet complexes ($CD14^{-}CD61^{+}$ within PMN gate). Data are expressed as the percentage of leukocytes associated with platelets ± SEM of four independent experiments. In **(b)** and **(d)**, each dot represents data corresponding to an individual donor. * *P-value* < 0.05.

**Figure 2. *Brucella abortus* promotes platelet–PMN complexes formation in whole blood.** Flow cytometry analysis of whole blood uninfected **(a)** or infected with *B. abortus* for 30 min **(b)**. Blood samples were stained with anti-CD61PE and anti-CD14PerCP antibodies and analyzed by flow cytometry in order to identify platelet-leukocyte complexes. **(c)** Quantification of platelet-PMN complexes within PMN gate. Data are expressed as the percentage of leukocytes associated with platelets ± SEM of three independent experiments. **(d)** CD61 expression in platelet-bearing leukocytes ($CD14^{-}CD61^{+}$ within PMN gate). Bars represent the arithmetic means ± SEM of three experiments. MFI, mean fluorescence intensity. **(e)** Confocal micrographs of PMN infected or not with *B. abortus* in presence of platelets for 30 min. Neutrophils were stained with an anti-human CD11b primary Ab follow by an anti-mouse IgG2aAlexa-546 secondary Ab (red). Platelets were stained with an anti-human CD61 primary Ab follow by an anti-mouse 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 platelets. Blue arrows indicate platelets bound to PMN while orange arrows indicate free platelets. PMN: PTL: *Ba* 1:100:100. *** *P-value* < 0.001 *vs*. Uninfected; NS: not significant.

**Figure 3. Platelets promote a *B. abortus*-mediated activation of PMN cells.** Isolated PMN cells were infected with *B. abortus* in presence of platelets for 30 minutes and activation of PMN was assessed by different techniques. **(a)** PMN supernatants were collected and IL-8 concentration was quantified by ELISA. PMN population was identified in a FSC *vs.* SSC dot plot. The surface

expression of CD11b **(b)**, CD62L **(c)** and ROS formation by Dihydrorhodamine 123 (DHR) fluorescence **(d)** was assessed by flow cytometry within this region. Bars represent the arithmetic means ± SD of five independent experiments, conducted with blood samples obtained from different donors. MFI, mean fluorescence intensity. PMN: PTL: *Ba* 1:100:100. ** *P-value* < 0.01; *** *P-value* < 0.001 *vs.* Uninfected. ### *P-value* < 0.001.

**Figure 4. Platelet-PMN interaction is required for ROS production but not for CD11b modulation.** Isolated PMN cells were treated with supernatants collected from *B. abortus*-infected platelets for 30 minutes and activation of PMN was assessed by flow cytometry. PMN population was identified in a FSC *vs.* SSC dot plot. The increase in the FSC parameter of PMN was assessed **(a and b)** and quantified **(c)**. The surface expression of CD11b **(d)** and ROS formation by DHR fluorescence **(e)** was also analyzed within this region. Bars represent the arithmetic mean ± SD of five independent experiments, conducted with blood samples obtained from different donors. ** *P-value* < 0.01; *** *P-value* < 0.001 *vs.* Untreated. ### *P-value* < 0.001. NS: not significant.

**Figure 5. Supernatant from *B. abortus*-infected platelets promotes PMN transmigration. (a)** Isolated PMN were seeded in the upper chamber of a *transwell* plate and supernatants collected from *B. abortus*, platelets or *B. abortus*-infected platelets were used as chemoattractant in the lower chamber. Results were expressed as percentage of migrated PMN relative to the seeded cells. **(b)** Isolated PMN were pre-treated for 30 minutes with supernatants collected from *B. abortus*, platelets or *B. abortus*-infected platelets. Afterwards, cells were washed and seeded in the upper chamber of a transwell plate and pleural effusion from tuberculosis patients 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 count by flow cytometry. Results were expressed as percentage of migrated PMN relative to the seeded cells. Bars represent the mean ± SD of duplicates from a representative experiment of at least three performed, conducted with blood samples obtained from different donors. * *P-value* < 0.05; *** *P-value* < 0.001 *vs.* Medium. ### *P-value* < 0.001. NS: not significant.

**Figure 6. Platelets promote *B. abortus* restraint and elimination by neutrophils.**
**(a)** Isolated PMN cells were infected with *B. abortus* in presence or absence of platelets at different ratios for 1 h and then incubated with antibiotics for 2 or 3 additional h. PMN were finally lysed and plated. **(b)** Isolated PMN cells were infected with *B. abortus* in presence or

absence of platelets at different ratios for 1, 2 or 3 h. Free cell supernatants were then plated. Data are expressed as Colony Forming Units (CFU) $mL^{-1}$ at each time point. Bars represent the mean ± SD of triplicates from a representative experiment of at least three performed, conducted with blood samples obtained from different donors. PMN: PTL: *Ba* 1:10:10 or 1:10:100 as specified. *** *P-value* < 0.001; ** *P-value* < 0.01 *vs*. PMN + *Ba*. ### *P-value* < 0.001 *vs*. *Ba*.

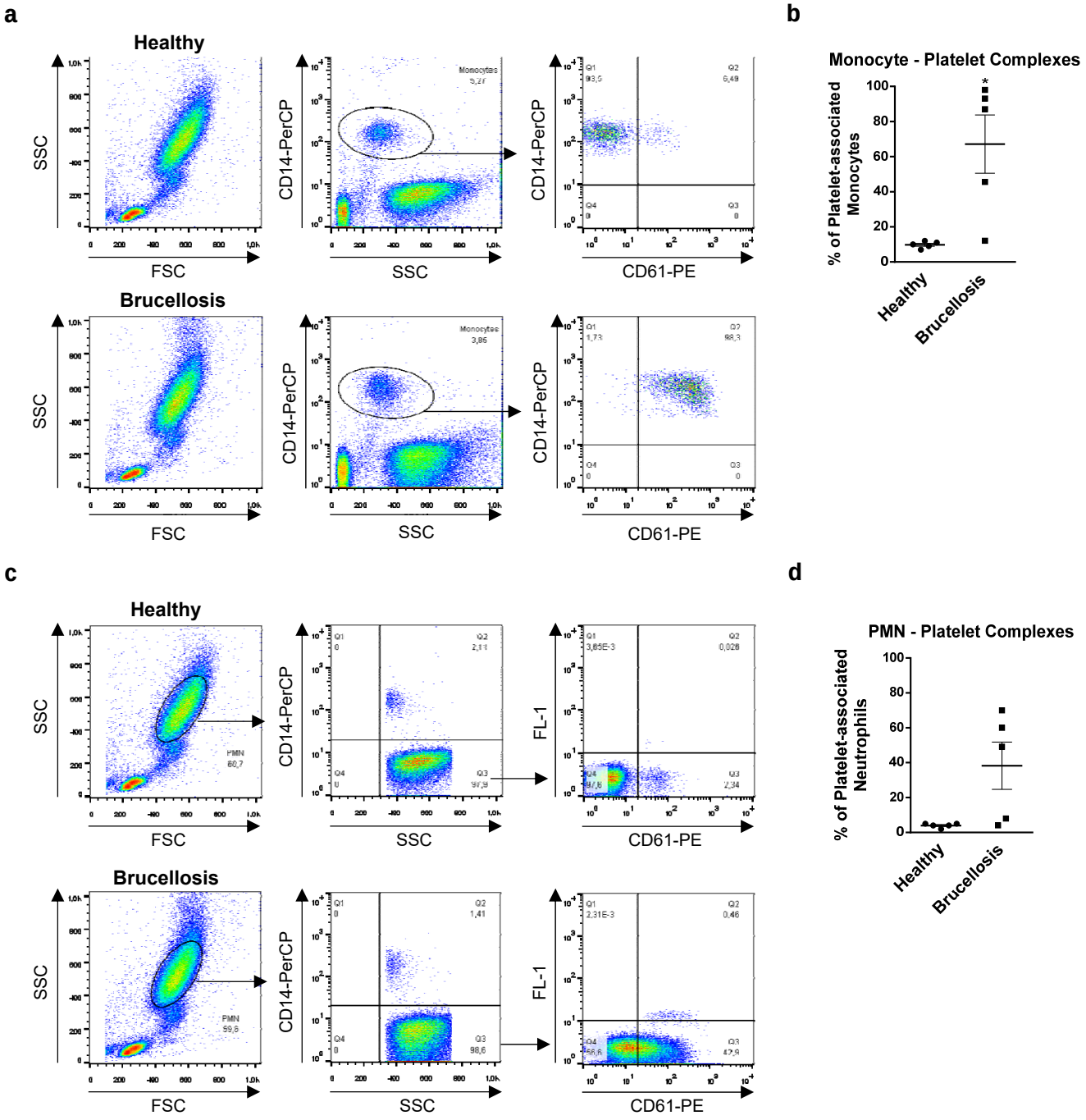

a
Healthy
SSC
FSC
CD14-PerCP
SSC
Monocytes
CD14-PerCP
CD61-PE
Brucellosis
SSC
FSC
CD14-PerCP
SSC
Monocytes
3,85
CD14-PerCP
CD61-PE
b
Monocyte - Platelet Complexes
% of Platelet-associated Monocytes
100
80
60
40
20
0
*
Healthy
Brucellosis
c
Healthy
SSC
FSC
PMN
60,7
CD14-PerCP
SSC
FL-1
CD61-PE
Brucellosis
SSC
FSC
PMN
59,6
CD14-PerCP
SSC
FL-1
CD61-PE
d
PMN - Platelet Complexes
% of Platelet-associated Neutrophils
100
80
60
40
20
0
Healthy
Brucellosis

**Figure 1**

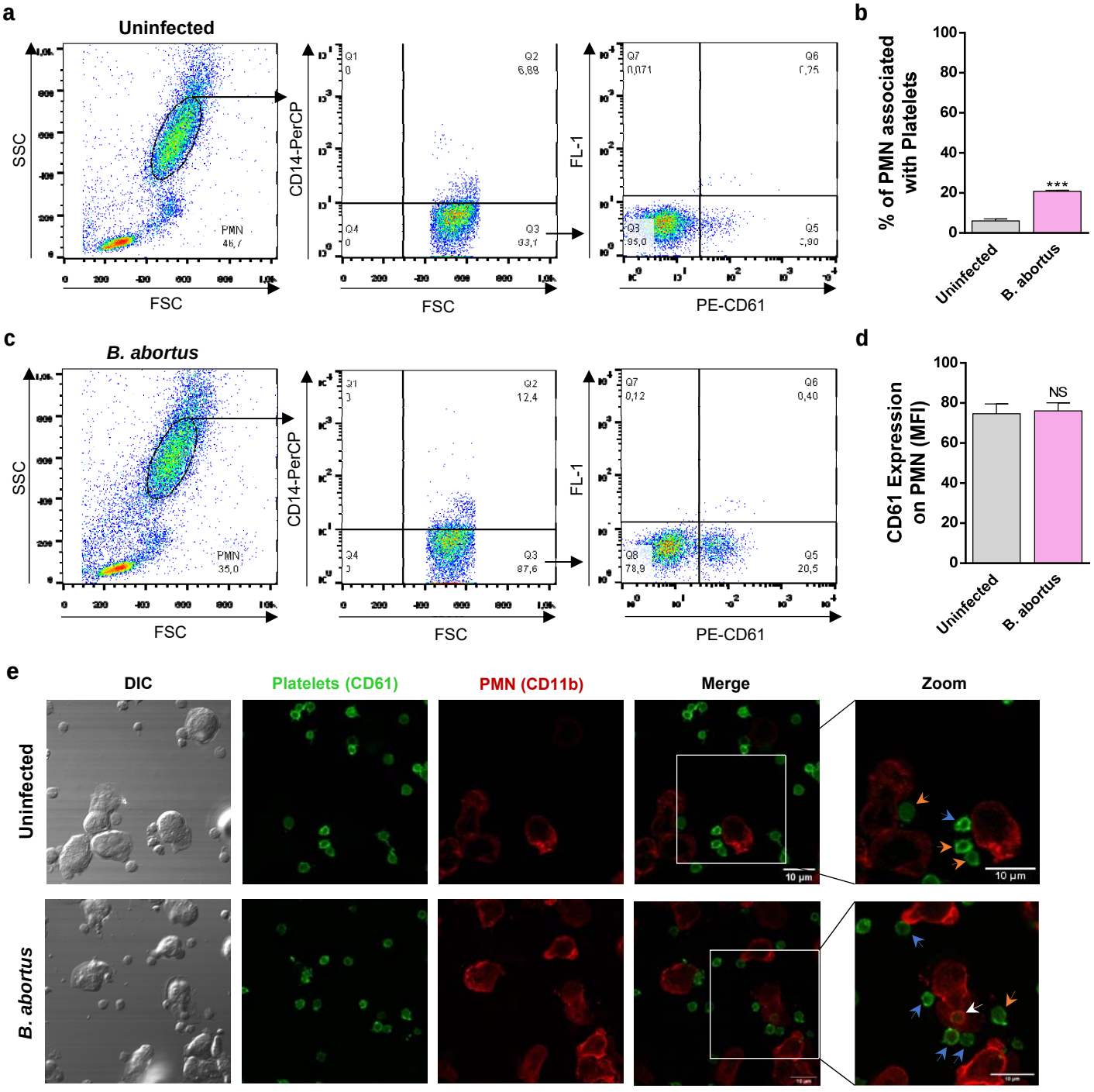

a
Uninfected
SSC
FSC
PMN
46,7
CD14-PerCP
Q1
0
Q2
6,89
Q4
0
Q3
93,1
FL-1
Q7
0,071
Q6
1,75
Q3
95,0
Q5
3,90
PE-CD61
b
% of PMN associated with Platelets
100
80
60
40
20
0
***
Uninfected
B. abortus
c
B. abortus
SSC
FSC
PMN
35,0
CD14-PerCP
Q1
3
Q2
12,4
Q4
3
Q3
87,6
FL-1
Q7
0,12
Q6
0,40
Q8
78,9
Q5
20,5
PE-CD61
d
CD61 Expression on PMN (MFI)
100
80
60
40
20
0
NS
Uninfected
B. abortus
e
DIC
Platelets (CD61)
PMN (CD11b)
Merge
Zoom
Uninfected
B. abortus
10 µm
10 µm

**Figure 2**

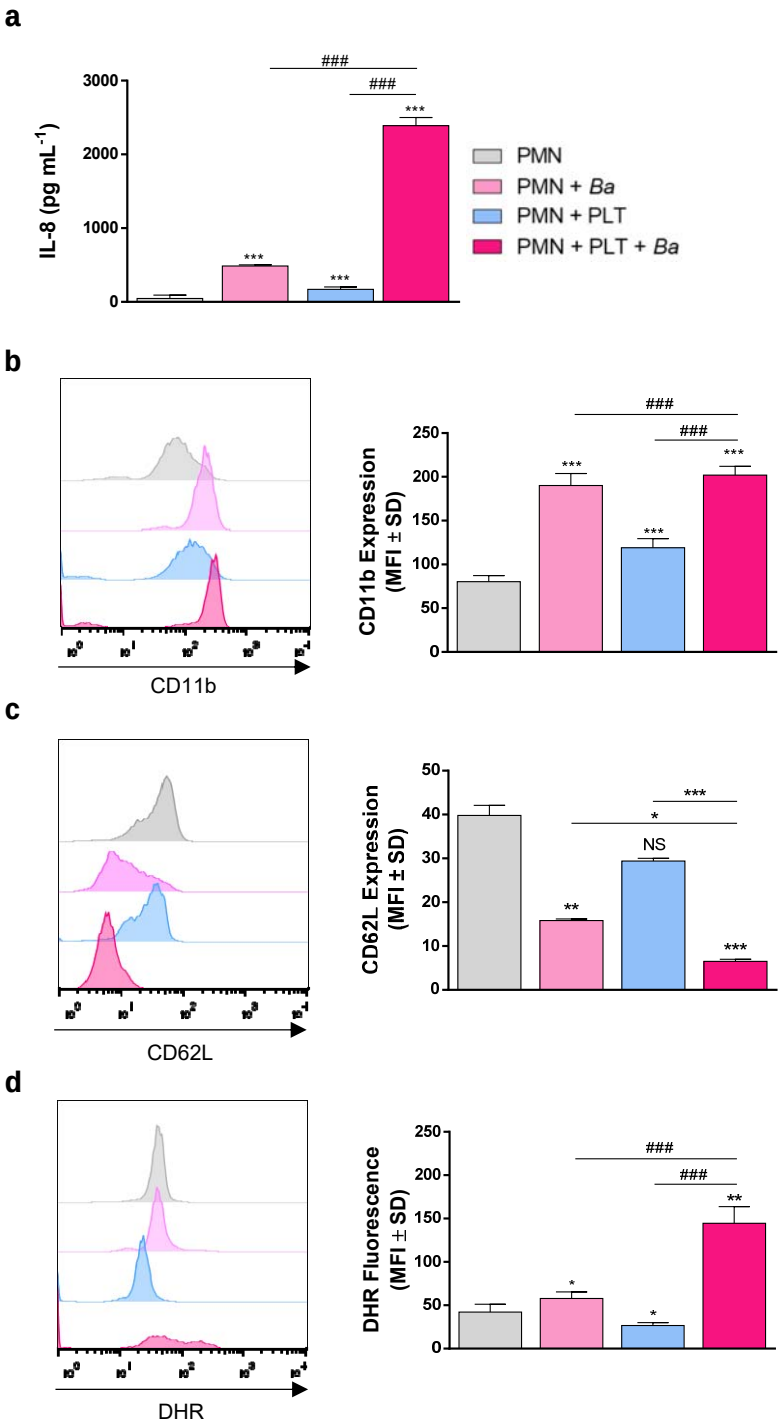

a
IL-8 (pg mL-1)
3000
2000
1000
0
###
###
***
***
***
PMN
PMN + Ba
PMN + PLT
PMN + PLT + Ba
b
CD11b
CD11b Expression (MFI ± SD)
250
200
150
100
50
0
###
###
***
***
***
c
CD62L
CD62L Expression (MFI ± SD)
50
40
30
20
10
0
***
*
NS
**
***
d
DHR
DHR Fluorescence (MFI ± SD)
250
200
150
100
50
0
###
###
**
*
*

**Figure 3**

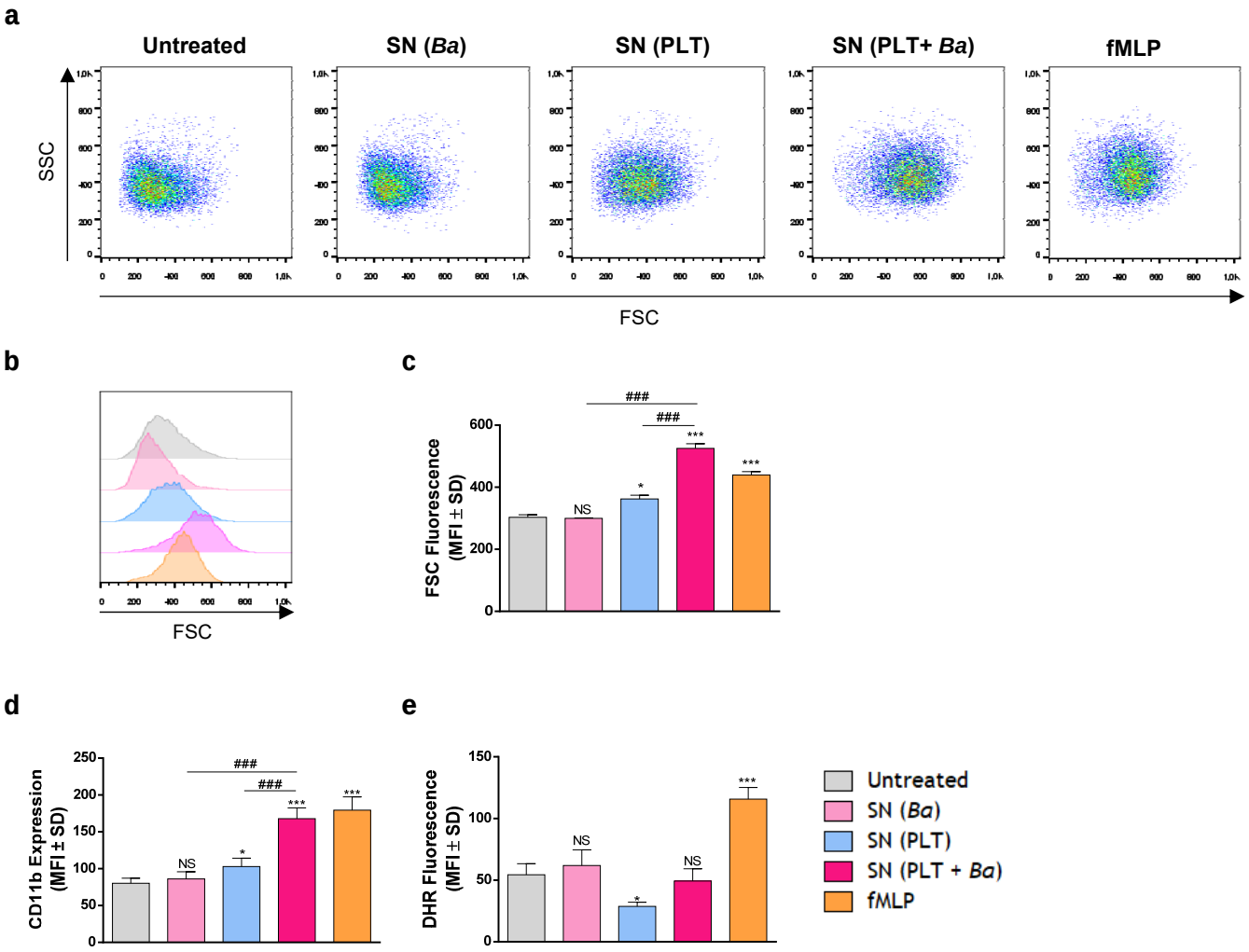
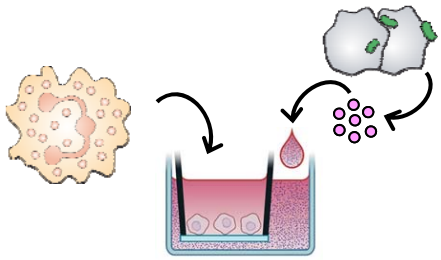
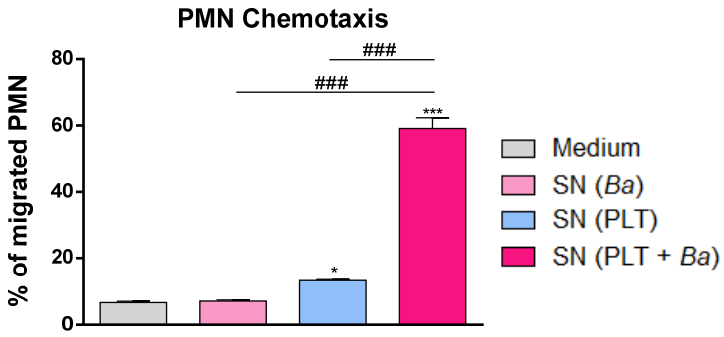

a
Untreated
SN (Ba)
SN (PLT)
SN (PLT+ Ba)
fMLP
SSC
FSC
b
FSC
c
FSC Fluorescence (MFI ± SD)
###
###
***
***
*
NS
d
CD11b Expression (MFI ± SD)
###
###
***
***
*
NS
e
DHR Fluorescence (MFI ± SD)
***
NS
*
NS
Untreated
SN (Ba)
SN (PLT)
SN (PLT + Ba)
fMLP

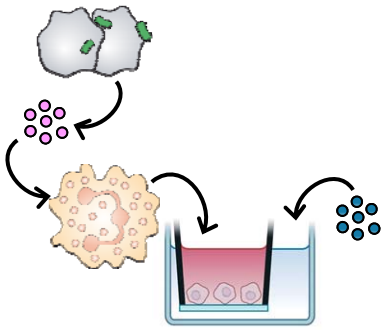
**Figure 4**

a




PMN Chemotaxis
% of migrated PMN
80
60
40
20
0
###
###
***
*
Medium
SN (Ba)
SN (PLT)
SN (PLT + Ba)

b



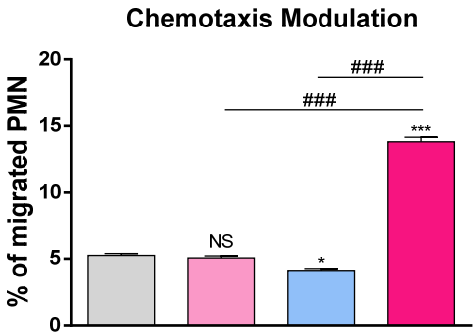

Chemotaxis Modulation
% of migrated PMN
20
15
10
5
0
###
###
***
NS
*

**Figure 5**

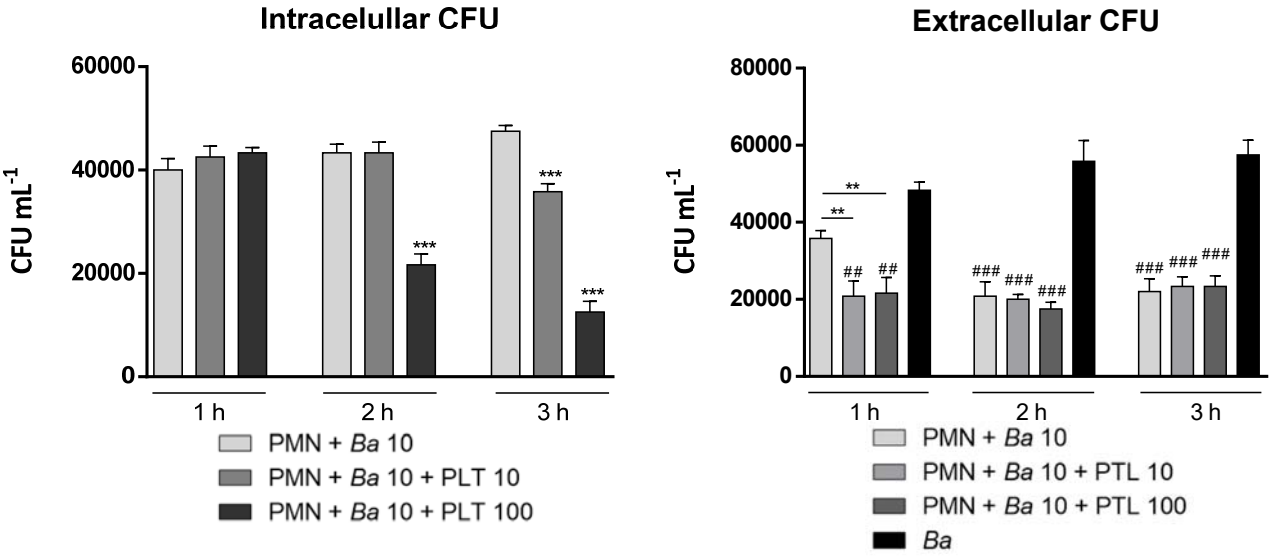

Intracelullar CFU
CFU mL-1
60000
40000
20000
0
***
***
***
1 h
2 h
3 h
PMN + Ba 10
PMN + Ba 10 + PLT 10
PMN + Ba 10 + PLT 100
Extracellular CFU
CFU mL-1
80000
60000
40000
20000
0
**
**
##
##
###
###
###
###
###
###
1 h
2 h
3 h
PMN + Ba 10
PMN + Ba 10 + PTL 10
PMN + Ba 10 + PTL 100
Ba

**Figure 6**