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Respuesta inmune producida por plaquetas durante la infección con *Mycobacterium tuberculosis*.

TESIS QUE PRESENTA

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PARA OBTENER EL GRADO DE DOCTOR EN CIENCIAS BIOMÉDICAS BÁSICAS

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Tesis que presenta:

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Dedicatoria

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Thank you,

Microbial Pathogenesis

Platelets immune response against *Mycobacterium tuberculosis* infection.

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Abbreviations used: platelets (PLT), activated platelets (A-PLT), *Mycobacterium tuberculosis* (Mtb), tuberculosis (TB), Host Defense Peptides (HDPs), human ß-defensin 2 (HBD2) and human-platelet factor 4 (hPF-4).

Abstract:

Tuberculosis (TB) is the first cause of death by a single infectious agent. Previous reports have highlighted the presence of platelets within Tb granulomas, albeit the immune-associated platelet response to *Mycobacterium tuberculosis* (Mtb) has not be deeply studied. Our results showed that platelets are recruited into the granuloma in the late stages of tuberculosis. Furthermore, electron-microscopy studies showed that platelets can internalize Mtb and produce host defense peptides (HDPs), such as RNase 7, HBD2 and hPF-4 that bind to the internalized Mtb. Mtb-infected platelets exhibited higher transcription and secretion of IL-1ß and TNF-α, whereas IL-10 and IL-6 protein levels decreased. These results suggest that platelets participate in the immune response against Mtb through HDPs and cytokines production.

Key words: Tuberculosis; platelets; immune response; host defense peptides.

1. Introduction

Mycobacterium tuberculosis (Mtb) is the causal agent of tuberculosis (TB), which is the leading cause of death from a single infectious agent; indeed, in 2018 TB caused 1.5 million deaths and infected 10 million people worldwide [1]. The success of this disease is because of its high transmissibility, since only one bacillus contained in secretions drops expulsed from patients with active disease is enough to infect close contacts [2]. Once an individual has inhaled the bacilli, these Mtb enter into the lungs encountering several immune cells such as macrophages [3], dendritic cells [4], lymphocytes [5], and epithelial cells [6]. Most of the infected people develop latent infection, whereas only 10% of these persons will develop active disease [7]; the outcome of Mtb infection will depend on the immune response of each person, in which platelets have not been taken into account as part of the mounted immune response hitherto.

In general, platelets have been underestimated in the immunity of most of the infectious diseases and TB is not the exception; but recently, platelets have been considered participants of the immune response because they have integrins and functional receptors all over their membrane, such as PARs, TLRs, DC-SIGN and C-type lectin-receptors [8, 9]. Platelets also express a huge number of biologically active proteins contained in their granules, such as cytokines, chemokines, coagulation and growth factors; these molecules can regulate the immune response produced in the infection site by immune cells [9]. Furthermore, platelets can respond to pathogens by releasing antimicrobial molecules as factor platelet 4 and defensins [10, 11], but their antimicrobial activity is dependent on their activation

state [12]; in the other hand, several reports have shown that platelets are capable to internalize pathogens [13], albeit this mechanism has not been well described.

There is scarce information regarding the role of platelets during Mtb infection, several reports have correlated the level of thrombocytosis with the severity of TB [14]. Indeed, some researchers have even suggested the use of this clinical parameter as a biomarker for treatment efficacy. More recently, platelets were found in lung biopsies from Tb patients, specifically in their granuloma structure [15], where platelets might be modulating the macrophages response to M2 phenotype [16], which is associated with increased mycobacterial survival. In spite of these reports, there is scant information regarding the direct interaction between platelets and Mtb, therefore the aim of this study was to study the direct platelets' response against Mtb.

2. Materials and methods

2.1 Mycobacterium tuberculosis Culture

Mycobacterium tuberculosis (Mtb) H37Rv strain (ATCC No. 25618) was grown in 25 cm^2 flask with 20 mL of 7H9 media (Beckton Dickinson, Franklin Lakes, USA) supplemented with 0.2% of glycerol, 0.05% tween-80 (Sigma-Aldrich, St Luis, USA) and 10% Oleic acid-Albumin-Dextrose-Catalase (OADC) enrichment media (Beckton Dickinson, Franklin Lakes, USA). Mtb culture was incubated for 12 days at 37°C in agitation, until it reached logarithmic phase. Then it was divided into working aliquots and stored at -20ºC until use.

2.2 Experimental model of tuberculosis in BALB/c mice and immunohistochemistry (IHQ).

Animal work was performed in accordance with Mexican national regulations on Animal Care and Experimentation (NOM 062-ZOO-1999). The experimental model of progressive pulmonary TB was described in detail elsewhere [17]; Briefly, male BALB/c mice 6–8 weeks age, were anesthetized in a gas chamber using 0.1 mL per mouse of sevofluorane, and each mouse was infected by endotracheal instillation with $2x10^5$ of live bacilli. Mice were maintained in vertical position until they have been spontaneously recovered. Infected mice were maintained in groups of five in cages fitted with micro-isolators. Mice were euthanized after 7 and 60 days post-infection by exsanguination. Lungs were perfused with OCT by intratracheal via and they were quickly transferred into liquid nitrogen. Sections of 10μ width were obtained from frozen lungs using a cryostat; then tissue was fixed in absolute ethanol

for 2 minutes and OCT was removed with water-washes. Endogenous peroxidase was quenched with 0.03% H_2O_2 in absolute methanol and non-specific antibody binding sites were blocked using mouse IgG Blocking reagent (Vector Laboratories, Burlingame, CA). Tissue sections were incubated with anti-CD41 rat monoclonal antibody dilution 1:100 (Abcam, Cambridge, UK) for 2 hours at room temperature, and afterward, it was incubated with HRP-anti-IgG rat antibody 1:250 (Abcam, Cambridge, UK) for 30 minutes and bounded antibody was detected using DAB and slides were counterstained with hematoxylin; finally, histology analysis was realized in Zeiss Axiovert microscope (Zeiss company, Jena, Germany).

2.3 Platelets isolation and activation.

Platelets were isolated from buffy coats obtained from the General Hospital #1-IMSS, Zacatecas, Mexico. Blood was transferred into 50mL tubes and centrifuged at 300g for 20 minutes. Subsequently, platelet-rich plasma (PRP) was collected and diluted vol 1:1 with activation-inhibition cocktail (EDTA 13mM, PGE1 0.7μM, and acetylsalicylic acid at 195 μM, diluted in citrate-dextrose buffer at pH 7.4); then, diluted plasma was added over density gradient at 1.063 gr/mL with Optiprep (Sigma-Aldrich, St Luis, USA). Subsequently, samples were centrifuged for 20 min at 350g and platelet fraction was recovered into 50 mL tubes and centrifuged at 800g for 20 minutes, to get the platelet pellet. Afterward, the supernatant was removed, and platelets were resuspended in 20 mL of Tyrode's buffer; platelet numbers were counted in Neubauer chamber and the platelet purity was ≈90-95% (determined by flow cytometry with CD41 marker).

Platelets were activated with thrombin (Sigma-Aldrich, St Luis, USA) at 0.03 IU/mL for 30 minutes at room temperature; this thrombin concentration was selected because it doesn't have any effect in metabolic viability (determined by Alamar blue, data not shown); but induced ≈50% platelet activation (evaluated by CD62P marker by flow cytometry, data not shown) and platelets without stimuli but with the same time of incubation were used as the control condition.

2.4 Platelets infection with Mycobacterium tuberculosis

Non-activated and activated platelets were incubated with *M. tuberculosis* at different multiplicity of infection (MOI) 100:1, 10:1, 5:1, and 1:1 (platelets: bacteria) at different times: 3, 18, and 24 hours at 37°C in a 5% CO2 atmosphere.

2.5 Ultrastructural studies

Infected and non-infected platelets ($1X10^8$ platelets) were incubated for 24 hours at 37°C in 5% CO2 atmosphere; then platelets were prepared for transmission electron microscopy (TEM). For conventional TEM, platelets were fixed with 1% glutaraldehyde dissolved in cacodylate buffer 0.1M (pH7) followed by 2% osmium tetroxide and dehydrated with decreasing alcohol treatment and embedded in Spur's resin (Electron Microscopy Sciences, Hatfield, PA). Then, thin sections (90nm) were contrasted with uranyl acetate, lead citrate, and examined with a FEI Tecnai transmission electron microscope (*FEI Company*, OR, USA).

Immune-electron microscopy was performed for host defense peptide (HDP) detection in platelets using the Mtb incubation conditions mentioned above. Platelets

were fixed with 1% glutaraldehyde dissolved in cacodylate buffer 0.1M (pH7) overnight. After that, platelets were dehydrated with decreasing alcohol concentration treatment and embedded in hydrosoluble resin LR-White (Electron Microscopy Sciences, Hatfield, PA). Then, thin sections (120nm) were incubated, with antibodies for detection of LL-37 (Santa Cruz Biotechnology, California, USA), hPF4 (Preprotech, NJ, USA), HBD2 1:50 and RNase7 1:50 (Santa Cruz Biotechnology, California, USA); for 24 hours at room temperature. Then, sections were washed and incubated with anti-rabbit, anti-goat, or anti-mouse antibodies (Sigma-Aldrich, St Luis, USA) for 90 min. Later, sections were contrasted with uranyl acetate and observed with FEI Tecnai transmission electron microscope (*FEI Company*, OR, USA).

2.6 Antimicrobial activity against Mycobacterium tuberculosis

After 24h or 72 h of Mtb incubation, the platelet's anti-mycobacterial activity was determined by counting the colony forming units (CFU). Briefly, Mtb infected platelets were centrifuged and resuspended in SDS 1% for 10 min, and then SDS activity was inhibited using BSA 20%. Then, serial dilutions were carried out for each condition and subsequently plated on a Petri plate with 7H10 medium. Plates were incubated for 14 days at 37°C, and bacterial growth was determined by counting the colony forming units.

2.7 RNA isolation, cDNA synthesis and qPCR

After 3-, 18-, and 24-hours post-infection, platelets were lysed with Trizol reagent and RNA was extracted by adding 40µL Chloroform-isoamyl alcohol (49:1). The aqueous phase was mixed with 50µL of cold isopropanol and incubated for 90 minutes at -80ºC. The RNA pellet was obtained after samples were centrifuged and washed with 200µL of ethanol 70%. RNA was resuspended in DEPC water. Reverse transcription was performed with 2µg of RNA, incubated with 1µM DT prime (Thermo Scientific, Lithuania, USA), 1X RT buffer, 1µM of each deoxynucleoside triphosphate (dNTP) and 100 UI RevertAid Reverse Transcriptase (Thermo Scientific, Lithuania, USA).

Quantitative polymerase chain reaction (qPCR) was performed using the LightCycler 480 (Roche, Mannheim, Germany) equipment using Ssofast evagreen supermix (Bio-Rad, California, USA), with 0.5µM primers for each selected gene: IL-10 (F-tgggggagaacctgaagac and R-ccttgctcttgttttcacagg), TNF-α (F-cagcctcttctccttcctgat and R-gccagagggctgattagaga), IL-1ß (F-tacctgtcctgcgtgttgaa and R-tctttgggtaatttttgggatct), camp (F-gccgctgattcttttgacat and R-aatcttctccccacctttgc), hPF4 (F- gaagaccacctcccaggtc and R- ccattcttcagcgtggcta), TGF-ß (F-gcagcacgtggagctgta and R-cagccggttgctgaggta). Relative expression was calculated using the Livak method $2^{-\Delta\Delta Ct}$ using the gene Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Epsilon (YwhaE) as housekeeping gene (F- aaatgccaaatatcgcgcctc and R-ctgtcagtgtgtttggactagaga). Previously YwhaE gene was validated as housekeeping gene for platelets using method $2^{-\Delta Ct}$ (supplementary figure No. 1).

2.8 Cytometric bead Assay (CBAs)

Platelets were infected as mentioned above, then supernatants were collected 24h post-infection and filtered with 0.22µm membrane to remove any possible bacteria. Subsequently, the supernatants were used to determine cytokines and chemokine levels by using CBA human inflammatory cytokines kit, CBA human chemokine kit and human TGF-β1 single Plex flex Set (Beckton Dickinson, Franklin Lakes, USA) according to the manufacturer instructions and evaluated with a FACS Canto II (Beckton Dickinson, Franklin Lakes, USA).

2.9 Statistics

Distribution was evaluated using the Kolmogorov-Smirnov test, followed by one-way-Analysis of variance (ANOVA) or a Kruskal-Wallis test according to data distribution. Data was evaluated in GraphPad Prism 6 software. $p<0.05$ was considered statistically significant.

3 Results

3.1 Platelets are involved in the immune response to *M. tuberculosis* infection in an *in vivo* model.

To assess whether platelets are involved within TB pathology, the platelet marker CD41 was evaluated in a murine model of TB infection as described above. First, unspecific binding was discarded incubating the pulmonary tissue only with the secondary antibody anti-rat-IgG (Figure 1A). Then immunostaining for CD41 was evaluated in lungs from infected and non-infected mice, observing that in some areas from non-infected control mice, CD41 immunostaining labelling was seen in platelet-shape cells located on the alveolar walls and into occasional capillary lumen (Figure 1B), similar results were observed in infected lungs after 7 days post-infection (Figure 1C), being more frequent in areas of alveolar-capillary interstitial inflammation (Figure 1C, inset). At 60 days post-infection there are extensive pneumonic patches, which show granular CD-41 immunostaining between the inflammatory cells and on the membrane of lymphocytes and macrophages (Figure 1D, inset). Interestingly, numerous cytoplasmic vacuoles with strong CD41 immunostaining were seen in some foamy macrophages located within pneumonic areas (Figure 1D).

3.2 Platelets can internalize *M. tuberculosis* independently of their activation stage.

Transmission electron microscopy was used to study the interaction between platelets and Mtb. Purified platelets were incubated for 24 hours with a MOI of 10.

Non-activated platelets show well preserved cytoplasmic granules (Figure 2A), confirming that platelets were not activated by our purification procedure (Figure 2A, inset). Extensive platelets degranulation was observed in platelets with previous activation (Figure 2C). Interestingly, Mtb incubated with non-activated or activated-platelets were found in the cytoplasm of platelets (Figure 2B and 2D, respectively), but Mtb internalization by non-activated platelets produced very limited degranulation (Figure 2B, inset). Thus, it seems that platelets are able to internalize Mtb without substantial changes in their activation stage or inducing extensive degranulation. To further assess these results, platelets were infected with PKH26-stained Mtb and platelets were immuno-stained with CD41 and percentage of infection was determined using flow cytometry. Results showed that platelets internalized actively Mtb independently of the activation state (Supplementary 4).

3.3 Platelets produce Host Defense Peptides that are associated with intracellular M. *tuberculosis*.

HDPs such as RNase 7, HBD2, and hPF-4 were localized by immunoelectronmicroscopy in non-activated, activated, infected and non-infected platelets. Non-activated and activated platelets show RNase 7 in their cytoplasm (Figure 3A), mainly around platelet's granules in activated platelets (Figure 3B). HBD2 showed similar immune-detection patterns than those of RNase 7 (Figure 3C, 3D). Peptide hPF-4 was abundantly expressed inside of granules in non-activated platelets (Figure 3E), and in the cytoplasm of activated platelets (Figure 3F). Thus,

non-infected platelets, activated or non-activated constitutively have RNase7, HBD2, and hPF4.

Subcellular HDPs immunodetection was then evaluated in infected platelets, both activated and non-activated. RNase 7 was co-localized with intracellular Mtb cell-wall either in non-activated or activated-platelets (Figure 4A and 4B, respectively). Similar results were observed with HBD2, which was localized in Mtb-infected platelets on Mtb cell-wall, and activated platelets showed higher labeling than non-activated platelets (Figure 4C and 4D, respectively). Finally, hPF-4 was found on the Mtb cell-wall in both experimental conditions, being higher in non-activated than in activated platelets (Figure 4E and 4F, respectively).

3.4 Platelets do not show anti-mycobacterial activity

To evaluate whether the expression of host defense peptides produced bacterial killing, both activated and non-activated platelets were incubated with different MOI for 24 (Figure 5A) and 72 hours (Figure 5) and CFUs were determined. In both experimental conditions there were not seen significant changes. Thus, it seems that platelets do not affect bacterial viability.

3.5 M. tuberculosis infection induces cytokine production in platelets.

Transcription of CAMP, IL-1ß, TGF-ß, hPF-4, and TNF-α mRNA was evaluated by RT-qPCR. Mtb infection did not induce any change in CAMP, TGF-ß, and hPF-4 transcription after 3, 18 and 24h post-infection (Figure 6A, 6C and 6D, S2). Interestingly Mtb infection induced significant higher mRNA expression of IL-1ß and

TNF-α in both non-activated and activated platelets only after 3h post-infection (Figure 6B and 6E, respectively, S2).

Cytokine concentrations were determined in supernatants by CBA human inflammatory cytokines/chemokines kit and cytofluorometry in both experimental conditions. Mtb infection induced significant higher IL-1ß secretion only in non-activated platelets (Figure 7A). In contrast, Mtb infection decreased IL-10 in both non-activated and activated platelets (Figure 7B), while IL-6 was significantly decreased in only non-activated platelets (Figure 7E). Mtb infection did not induce any change in the production of TNF-α (Figure 7C and 7D) and TGF-ß (Figure S3 B), neither in chemokines such as IL-8, IP-10, MCP-1, and MIG (Figure S3 A, C and D).

4. Discussion

Platelets have been underestimated in the immunology of TB. In the last few years, some reports regarded to the role of platelets in TB pathology have been published [16], but most of these studies described the interaction of platelets with other immune cells, mainly macrophages and its possible role to switch its phenotype to foamy macrophages [15, 18]. Nonetheless, none of them studied the direct immune effect of platelets against Mtb. Considering that platelets have been defined as immune cells because of their functional pathogen pattern recognition receptors expressed all over their membrane [19], it is likely that they can interact with pathogens and therefore develop an autonomous response against Mtb. Thus, in the present study was evaluated the specific platelet response against Mtb infection using purified platelets instead of platelet-rich plasma, as described elsewhere [20]. The presence of platelets has been described in granulomas from TB patients [15], but their function within the granuloma needs to be further determined. In the present study, the presence of platelets was evaluated in a well-characterized model of progressive TB in mice by the detection of the specific marker CD-41. Previous studies have reported the scarce presence of platelets in healthy lung tissue [21], similar to the results obtained in the present study; furthermore early Mtb infection, after one week, also showed occasional platelets on the alveolar epithelium. In contrast, CD-41 immunostaining that denotes platelets presence was abundantly detected during late disease in our mouse TB model, exhibiting a granular pattern around the inflammatory cells in the pneumonic areas, and in the cytoplasm of foamy/vacuolated macrophages, which could be due to platelets exposition of

phosphatidylserine on its membrane after their activation, considering that this molecule is recognized by macrophages to remove platelet debris. Indeed, other studies have reported that platelets phagocytosis by macrophages leads to a Mtb-anergic foamy macrophage phenotype (15). Our results contrast with previous studies that consider platelets as the first recruited cells into the sites of infection [8], but is in agreement with the presence of platelets during late disease, when extensive inflammation and tissue damage is produced by the strong immune response against Mtb [17]. It has been reported that during Mtb infection there is an increment in CXCL12 expression [22], and the receptors for this chemokine (CXCR4 and CXCR7) are expressed by platelets [23, 24]. Thus, the platelets recruitment observed during late experimental TB could be induced through these receptors; although it cannot be discarded that other chemokines could participate, since several chemokine receptors have been found in platelets.

Once it was determined that platelets arrived at the infection site on the late phase of murine TB model, it was assessed whether platelets interact directly with Mtb. Our ultrastructural studies revealed that platelets are capable to internalize Mtb, though the internalization mechanisms need to be further elucidated. Bacterial internalization by platelets has been previously described for *Staphylococcus aureus* but it had not been reported for Mtb [25]. Mtb size is very similar to platelets, and it is remarkable how platelets can internalize something bigger than themselves. Mtb internalization could be possible in platelets through their membrane system, this system can extend the platelets' membrane until cover latex particles that are bigger

than platelets size [26]. Thus, it seems that Mtb size is not a limiting factor to be internalized by platelets.

Platelets have several phagocytic receptors that are usually expressed by "professional" phagocytic cells, such as DC-SIGN [27], TLR-4 [28] and complement receptors (CR). Thus, platelets can use these receptors to internalize Mtb. After internalization, professional phagocytic cells promote bacterial elimination by ROS, NOS or HDPs production [29], since the production of ROS and NOS by platelets has been documented but their functionality has not been completely demonstrated [30]; we determined whether platelets with Mtb internalized were able to produce HDPs. Our ultraestructural results demonstrated that HDPs such as Rnase 7, HBD-2, and hPF-4 were produced by platelets during Mtb infection; and all of them interacted with Mtb-wall, thus we evaluated whether platelets were able to kill mycobacteria. Interestingly, the CFU determinations in platelets incubated with Mtb showed that notwithstanding the production of HDPs, platelets do not eliminate Mtb. In contrast, other studies have demonstrated the antimicrobial activity of platelets against diverse organisms, such as Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis*, *Candida albicans*, *Streptococcus agalactiae* and *Streptococcus oralis [31]. Perhaps the* more complex Mtb cell-wall structure confers higher resistance against HDPs or their concentration was not enough to achieve an efficient bacterial killing. However, other studies have reported that HDPs have immunomodulatory activity in lower concentrations [32]. Therefore, it is likely that platelets' role in TB could be more related to immunomodulation than to antimicrobial activity.

Since HDPs and TB infection by itself induce the production of cytokines and chemokines, we sought to determine the production of some of these molecules. mRNA levels of several cytokines were determined and only IL-1ß and TNF-α showed an increment induced by Mtb infection. Indeed, platelets do not have mRNA production *de novo,* nonetheless*,* there are reports of mRNA expression in platelets; being this immature and transferred from megakaryocytes [33]. RNA splicing machinery is present in platelets and most of their proteins are involved in the mRNA expression [34].

Determination of cytokines in supernatants collected from wells where platelets were incubated with Mtb confirmed the increase of IL-1ß production in co-existence with lower IL-10 concentrations, suggesting that Mtb induced a pro-inflammatory profile of cytokines through platelets response. Although, IL-6 which is a pro-inflammatory cytokine was also decreased. It is important to consider that IL-6 soluble receptor (IL-6sR) is also produced by activated platelets [35] and could capture IL-6, resulting in a decreased level of this cytokine. Nonetheless, further studies are need to elucidate this.

5. Conclusion

Platelets participate in TB immunity mainly in the late stage of the infection. Interestingly, Mtb can be internalized by platelets and induce an increase (IL-1ß) or decrease (IL-10 and IL-6) of cytokines secretion.

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7.- Foundings

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8. Conflict of Interest

Authors declare no conflict of interest

9. References

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Figure legends

Figure 1. **Representative micrographs of CD41 platelet marker detection by immunohistochemistry in lungs sections from Mtb-infected and non-infected mice.** A) There is not unspecific labeling by the incubation with the secondary anti-rat-IgG avoiding primary antibody in non-infected lung tissue. B) Non-infected lung showed positive CD-41 immunostaining labeling in platelet-shaped cells located on the alveolar epithelium surface (arrow) and into the capillary vessels (asterisk). C) After 7 days of infection, more numerous platelet-shape cells showed CD41 immune-staining (arrow). D) After 60 days of Mtb infection, diffuse interstitial CD-41 immunostaining was seen in the pneumonic patches (inset); high power magnification of these areas show granular CD-41 immunostaining between and on the surface of lymphocytes and macrophages, as well as in the cytoplasm of vacuolated macrophage (arrow). All micrographs magnification 630X and insets at 400X.

Figure 2. **Representative electron-microscopy micrographs of platelets incubated or not with M. tuberculosis.** A) Light power (inset) and high power micrographs showing well preserved granules in non-activated and non-infected platelets. B) Mtb incubated (MOI 1/10 during 24hr) with non-activated platelets show intracellular bacillus (asterisk). C) Non-infected activated platelets show degranulation with cytoplasmic extensions. D) Activated platelet show intracellular bacillus with small and numerous vacuoles (asterisk). Low power inset micrographs 16,500X.

Figure 3. **Representative micrographs of HDPs detection by immunoelectron microscopy in infected and activated or non-activated platelets.** Non-activated (A, C and E) and activated platelets (B, D and F) were processed for immunoelectron microscopy. RNase 7 (A and B), HBD2 (C and D) and hPF-4 (E and F) were immunolabeled (black dots, white arrows). Insets show whole platelet at 30 000X. magnification.

Figure 4. **Representative micrographs of the HDPs interaction with *M. tuberculosis* in activated and non-activated infected platelets.** Non-activated platelets (A, C and D), and activated platelets (B, D and F) were infected with Mtb at a MOI of 10 and incubated for 24h, afterward platelets were processed for immune-electron microscopy to detect RNase 7 in non-activated (A) and activated-platelets (B), HBD-2 in non-activated (C) and activated-platelets (D), and hPF-4 in non-activated (E) and activated-platelets (F). Asterisk (*) indicate the bacteria inside of platelets and arrows are pointing the peptide interaction with Mtb, which not show structural abnormalities. Insets show the whole platelet with intracellular Mtb.

Figure 5. **Platelets do not have a significant anti-mycobacterial activity.** Activated and non-activated platelets were incubated with Mtb at the indicated MOI for 24h (A) and 72h (B). Platelets were lysed to release internalized mycobacteria and Colony Forming Units (CFUs) were determined. Graphs show the results from 5 donors by duplicate.

Figure 6**. Gene transcription of cytokines and chemokines in infected or non-infected activated and non-activated platelets.** RT-qPCR analysis was carried out in both non-activated (PLT) and activated platelets (A-PLT), and both conditions incubated or not with Mtb. After 3 hours post incubation, platelets were lysed for RNA isolation and cDNA synthesis. Then, qPCR was performed using specific primers for the indicated gene. YwhaE was used as housekeeping gene to evaluate relative expression. *$p<0.05$ and **$p<0.01$ were considered as statistical significance. Graphs show the results from 3 donors by duplicate.

Figure 7. **Determination of cytokines in the supernatants of M. tuberculosis incubated with activated and non-activated platelets.** Supernatants were collected from non-activated (PLT) and activated-platelets (A-PLT) incubated with or not with Mtb. Incubation was carried for 24 h at a MOI of 10, the indicated cytokine level was evaluated using CBA human inflammatory cytokines kit. Graphs show the results from 8 donors. *$p<0.05$ and **$p<0.01$ were considered as statistical significance.

Figure 1

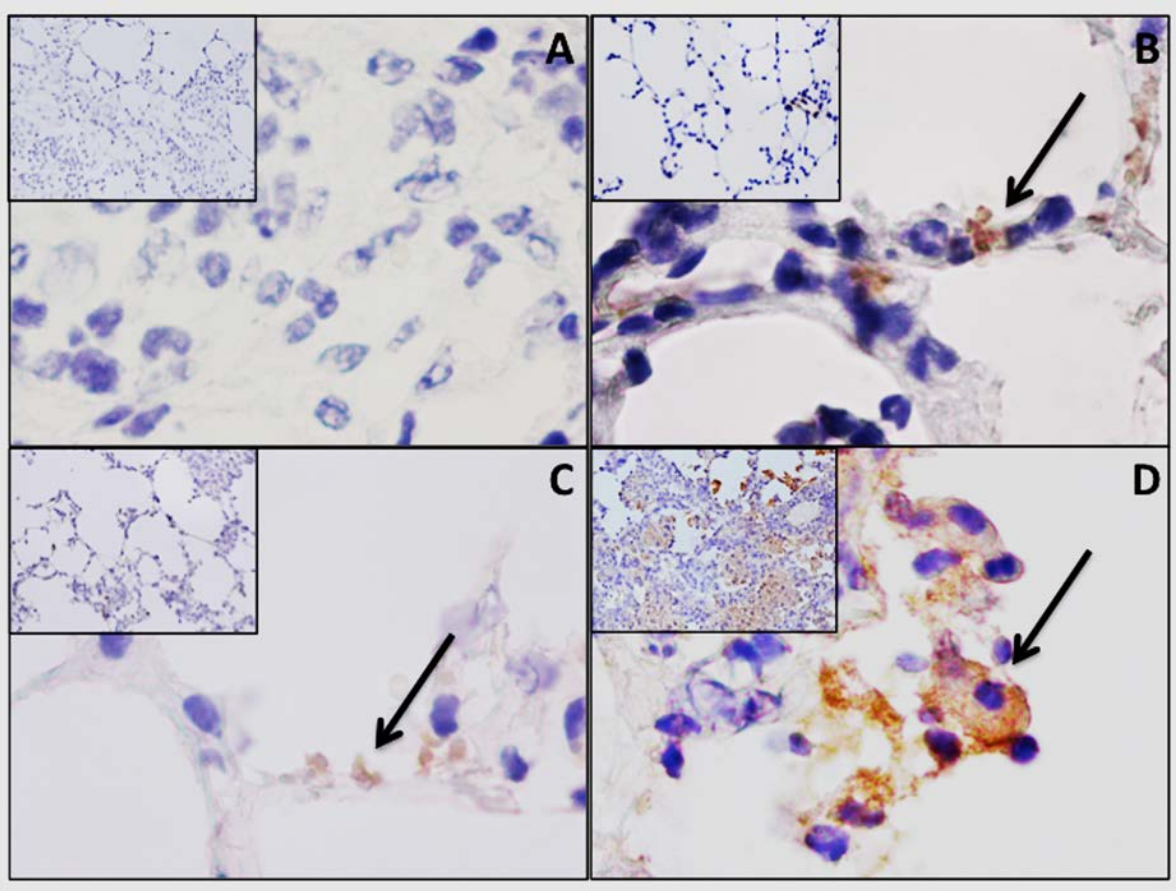


Figure 2

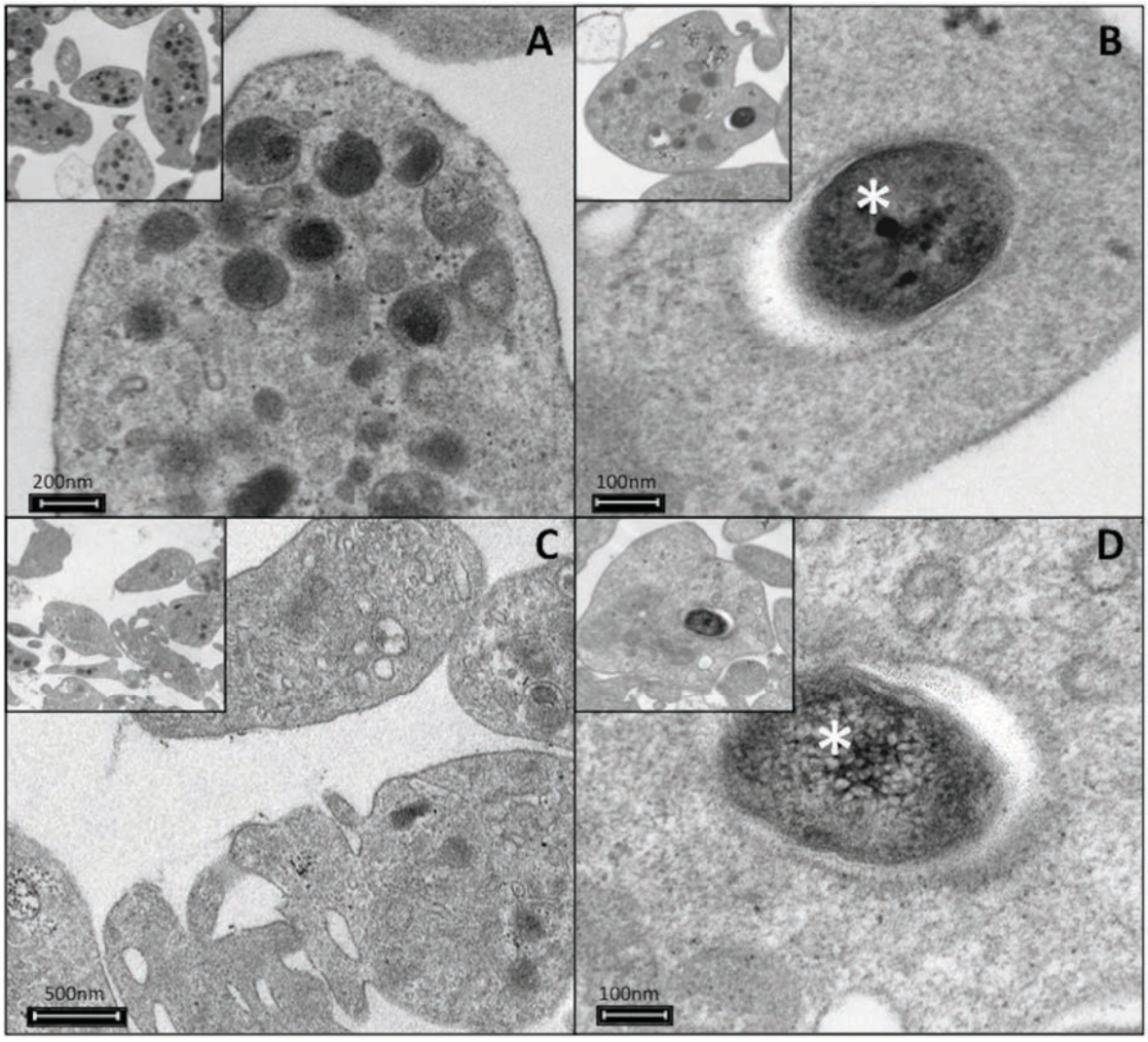


Figure 3

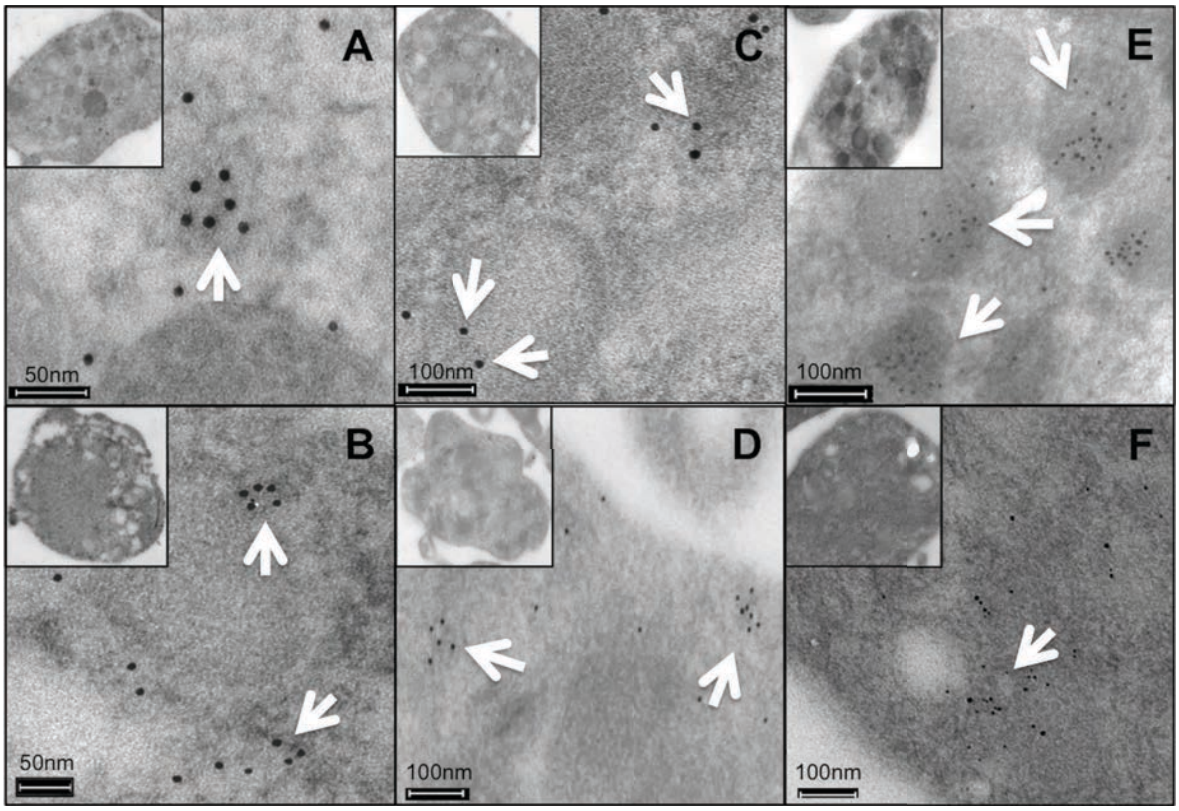


Figure 4

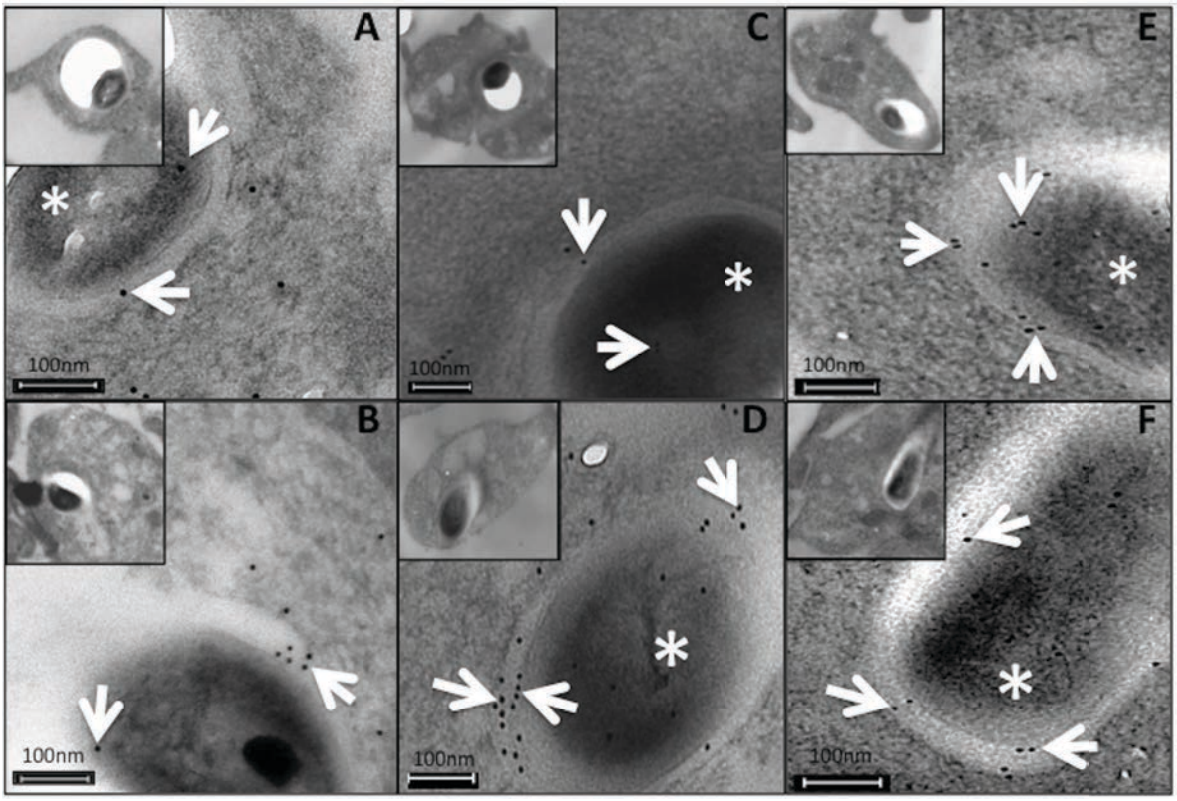


Figura 5

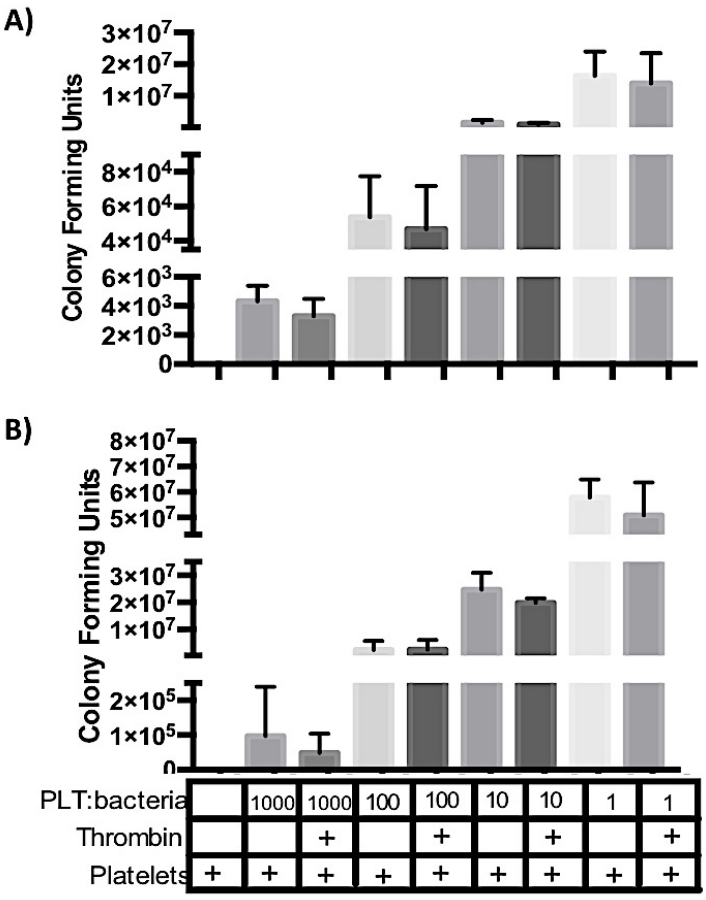

A)
Colony Forming Units
3×10^7
2×10^7
1×10^7
8×10^4
6×10^4
4×10^4
6×10^3
4×10^3
2×10^3
0
B)
Colony Forming Units
8×10^7
7×10^7
6×10^7
5×10^7
3×10^7
2×10^7
1×10^7
2×10^5
1×10^5
0
PLT:bacteria
1000 1000 100 100 10 10 1 1
Thrombin
+ + + + + +
Platelets
+ + + + + + + + +

Figure 6

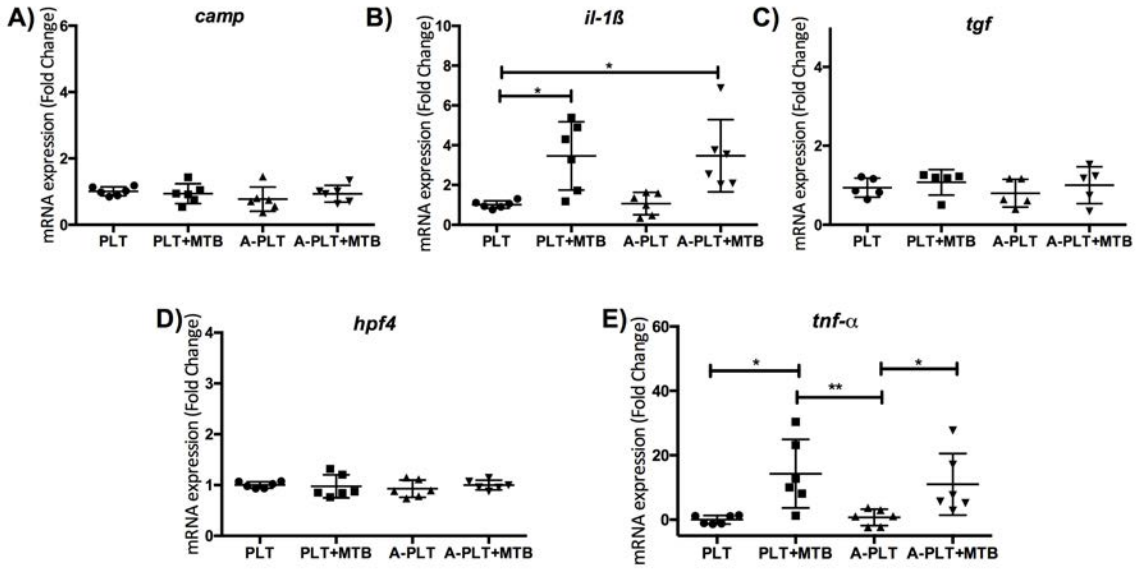
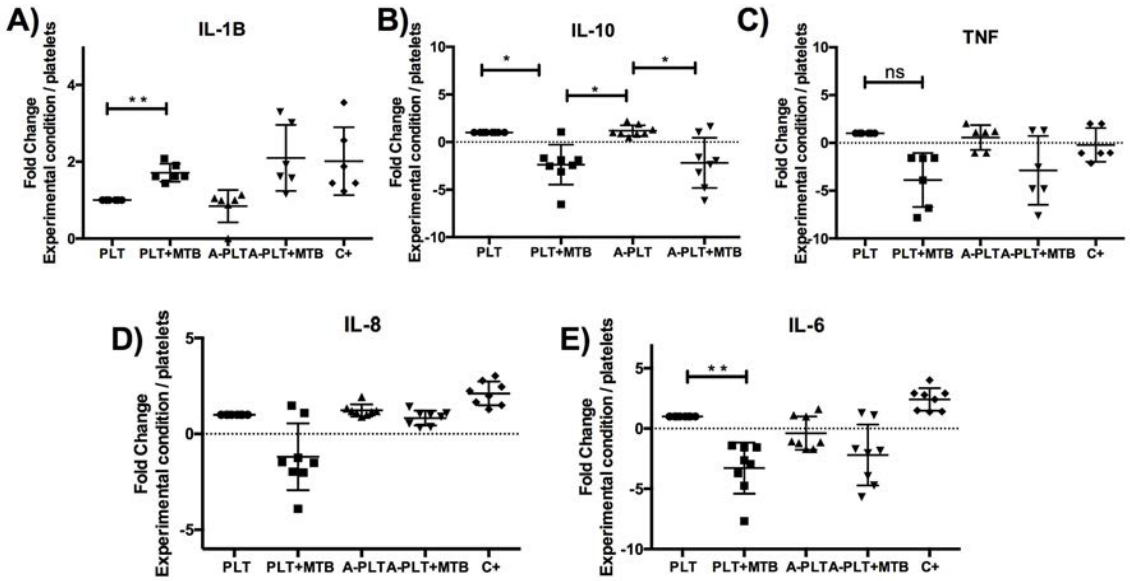
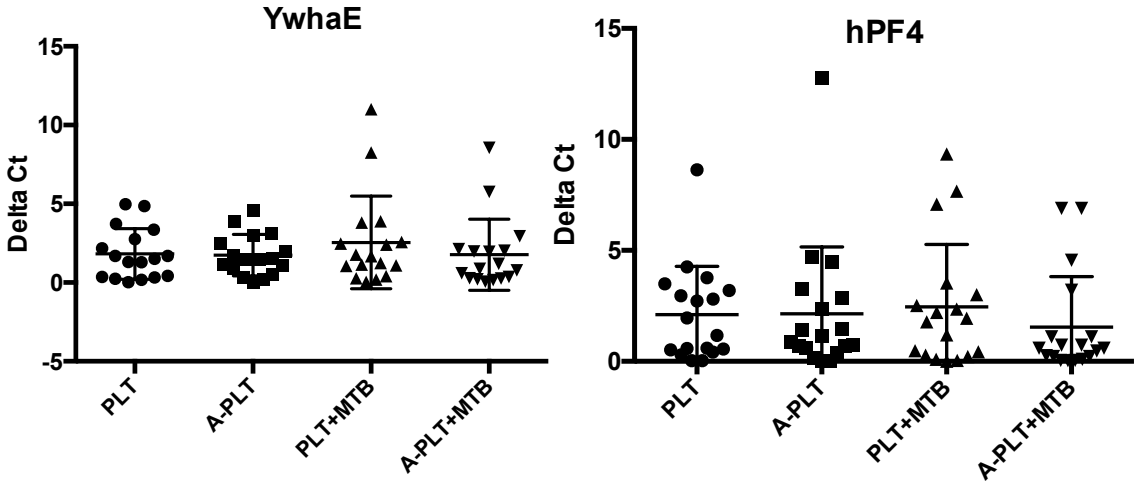

A)
camp
mRNA expression (Fold Change)
PLT
PLT+MTB
A-PLT
A-PLT+MTB
B)
il-1ß
C)
tgf
D)
hpf4
E)
tnf-α

Figure 7


A)
IL-1B
Fold Change
Experimental condition / platelets
**
PLT
PLT+MTB
A-PLT
A-PLT+MTB
C+
B)
IL-10
*
*
*
C)
TNF
ns
D)
IL-8
E)
IL-6

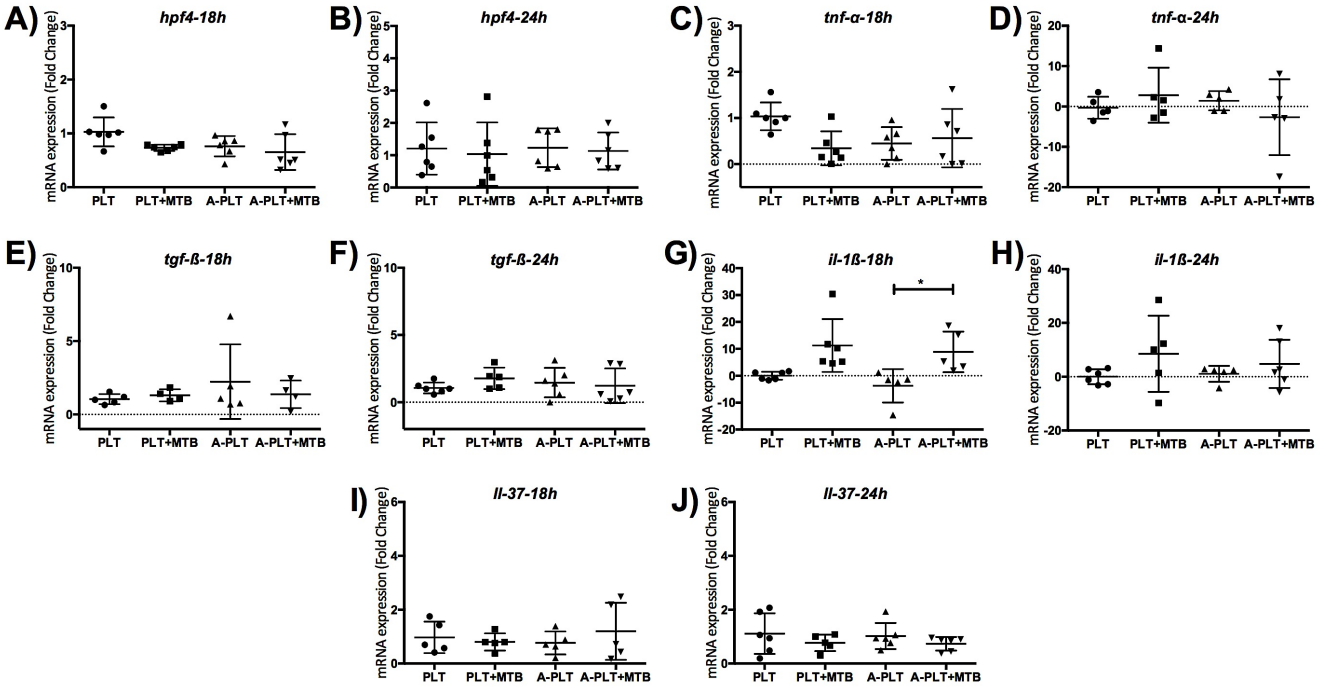
Supplementary figures

S1



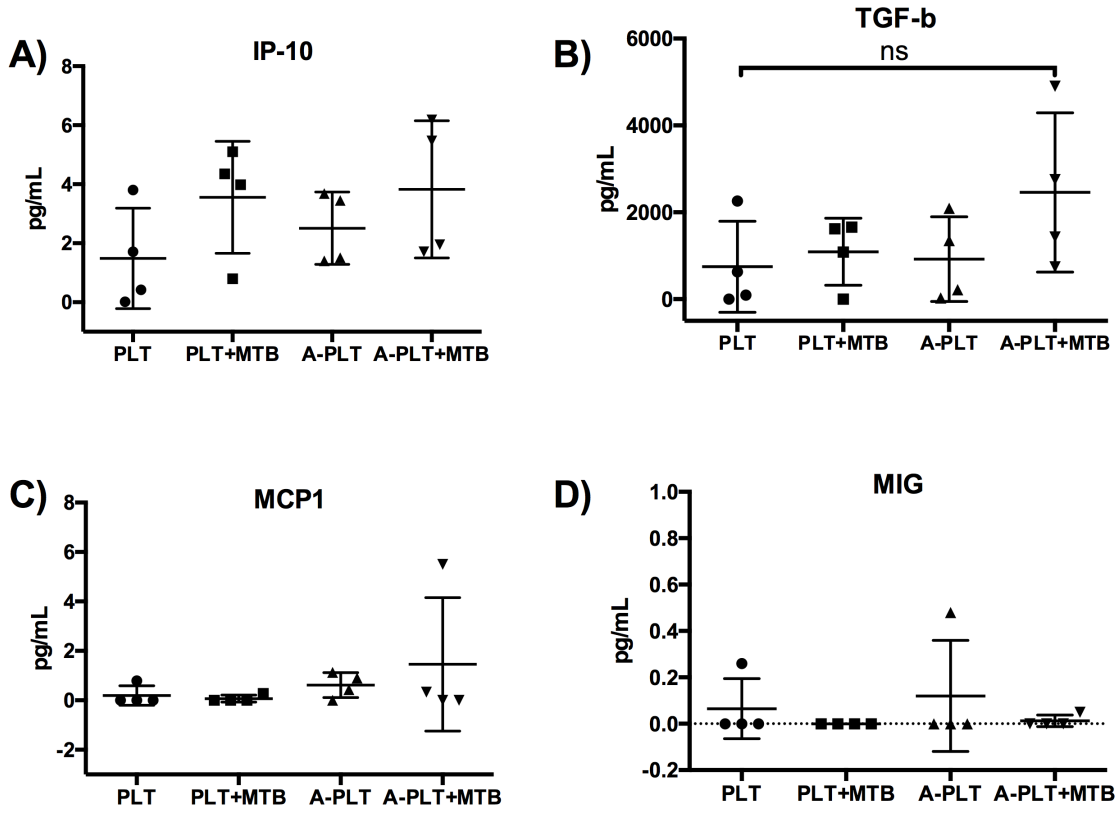
S1. **Validation of YwhaE gene as housekeeping.** RT-qPCR analysis was carried out in non-activated, activated platelets and both conditions with Mtb infection. After 3 hours post-infection, platelets were lysed for RNA isolation and cDNA synthesis. Afterward, qPCR was performed using specific primers for gene (YwhaE or hPF-4). Validation was realized using Livak method $2^{-\Delta Ct}$; anyone of genes evaluated have statistical significance between experimental conditions, however, YwhaE have less dispersion than hPF-4 and for that YwhaE was selected as housekeeping. Graphs are showing the results from 9 independent experiments by duplicate.

S2



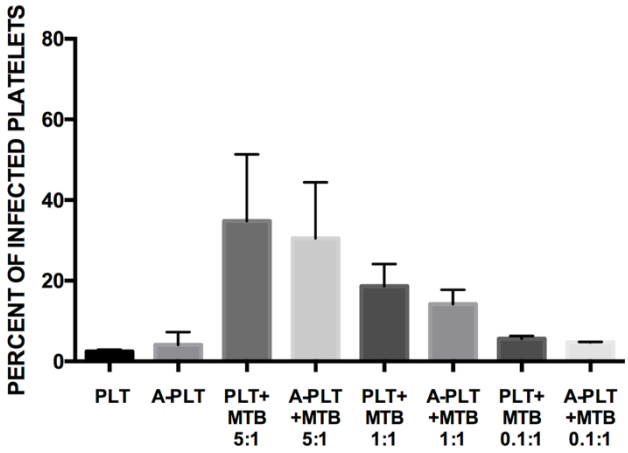
S2. ***M. tuberculosis*** **change mRNA expression in platelets at 18h and 24h post-infection**. RT-qPCR analysis was carried out in non-activated and activated-platelets, both conditions with Mtb infection. After 3 hours post infection platelets were lysed for RNA isolation and cDNA synthesis. Afterward qPCR was performed using specific primers for each gene to evaluate TNF-α, IL-1ß, CAMP, hPF4, and TGF-ß. YwhaE was used as housekeeping gene to evaluate their relative expression using Livak method $2^{-\Delta\Delta Ct}$. *$p<0.05$ was considered as statistical significance. Graphs show the results from 3 donors by duplicate.

S3



S3. ***M. tuberculosis*** **effect over TGF-ß and chemokines levels released by platelets.** Supernatants were collected from non-activated and activated-platelets, both with Mtb infection at MOI 10:1 for 24h. Chemokine levels were evaluated in supernatants using CBA human chemokine kit and human TGF-β1 single Plex flex Set according to its manufacturing instruction. Graphs show the results from 4 donors.

S4



S4. **Percentage of Mtb-internalization by platelets**. Activated and non-activated platelets were infected with different MOI for 24 h to determine the percentage of Mtb internalization. Results showed that platelets internalized Mtb even at a MOI of 0.1:1. Percentage for MOI of 5:1 was 34.83±16.49 for non-activated platelets (PLT), whereas for activated platelets (A-PLT) with the same MOI was 30.53±13.85. For MOI of 1:1 was 18.65±5.5 for PLT and 14.18±3.5 for A-PLT. For the case of 0.1:1 PLT showed 5.6±0.7 and A-PLT 4.7±0.14. Mean±SD. There were not statistical significance when coapred between PLT vs A-PLT for all MOI.

Universidad Autónoma de San Luis Potosí
Facultad de Medicina
Posgrado en Ciencias Biomédicas Básicas

Defensa de tesis

Respuesta inmune de las plaquetas durante la infección con *Mycobacterium tuberculosis*.

Presenta
M C Flor de María Torres Juárez

UIBMZ
FACULTAD DE MEDICINA UASLP

1

Comité tutelar

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Asesor Externo:

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Laboratorio adscrito:
Unidad de Investigación Biomédica de Zacatecas

2

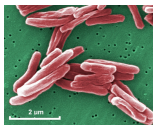
World Health Organization

Tuberculosis

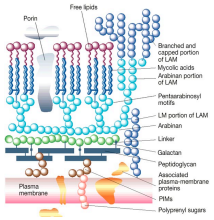
Enfermedad infecciosa que se presenta principalmente a nivel pulmonar causada por el bacilo *Mycobacterium tuberculosis*.

3

Mycobacterium tuberculosis



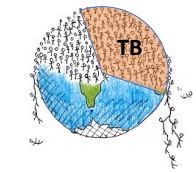
Janice Haney Carr. CDC (2015)



Park S. y Bendelac A. **Nature** (2000)

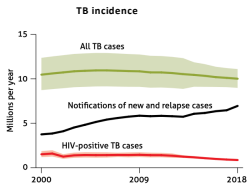
4

Tuberculosis en el mundo



10 million people fell ill with TB globally

1.5 million people died



Global Tuberculosis Report 2019, WHO

5

Tuberculosis en Mexico

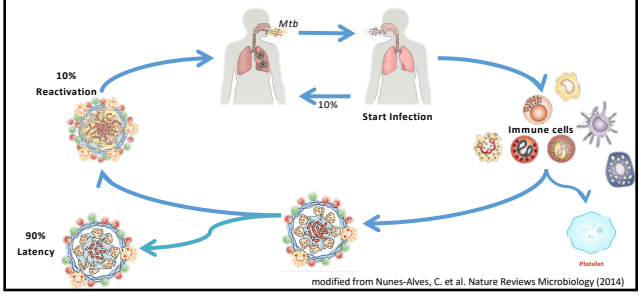
Incidencia de TB en Mexico

Casos de TB en América (2017).

Nº	COUNTRY	ESTIMATED CASES
1	Brazil	91,000
2	Peru	37,000
3	Mexico	28,000
4	Haiti	20,000
5	Colombia	16,000
6	Venezuela	13,000
7	Argentina	12,000
8	Bolivia	12,000
9	United States	10,000
10	Ecuador	7,200
Rest of the Americas		35,800
Total		282,000

6

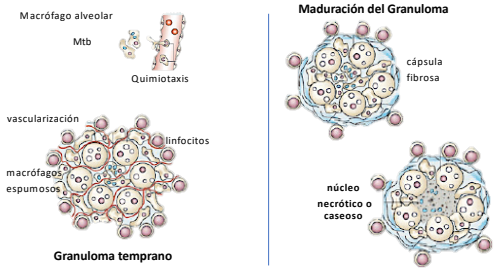
CICLO INMUNOLÓGICO DE TB



modified from Nunes-Alves, C. et al. Nature Reviews Microbiology (2014)

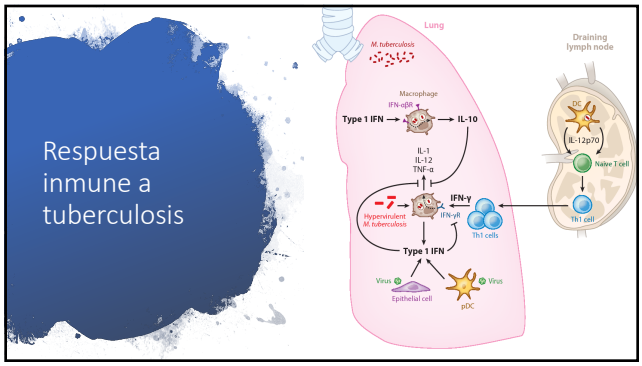
7

Formación del granuloma



Russell, D. Nature immunology (2009)

8



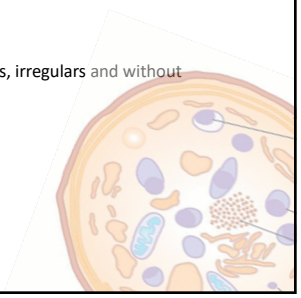
9

Platelets

Definition:

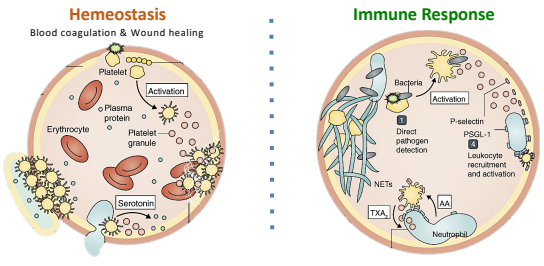
- Small cytoplasmatic fragments, irregulars and without nuclei.

"Platelets are the second most abundant component in blood."



10

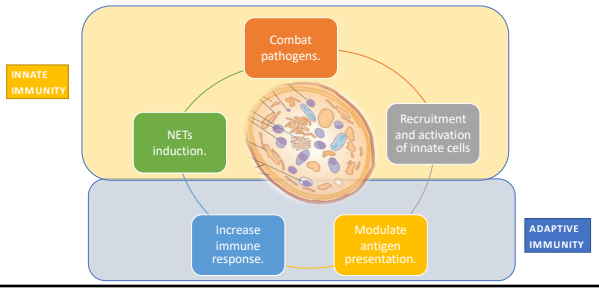
Platelets function



Jackson LiangYao Li, et al. JEM (2017)

11

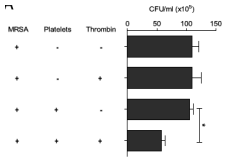
Platelet function in **Immunity**



12

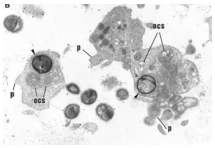
REPORTE DE PLAQUETAS EN INFECCIONES

- Antimicrobial effect



Ramadan A. Ali. Journal of Immunology (2016)

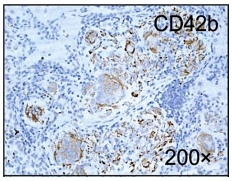
- Internalization of bacteria



Youssefian, T. Blood. (2002)

13

¿Han sido estudiadas las plaquetas en TB?

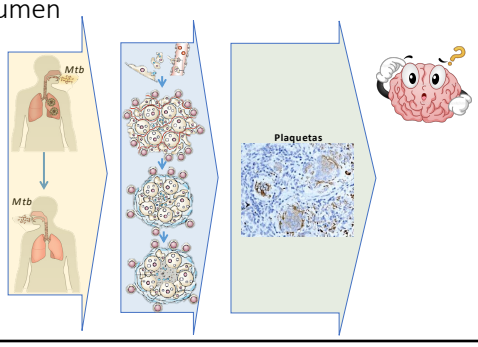


Biopsia de pulmón
Pacientes con diagnóstico de tuberculosis
Platelets= Immunohistochemistry CD42b+ hematoxylin

Feng Y. et al. J Infect Dis. (2014)

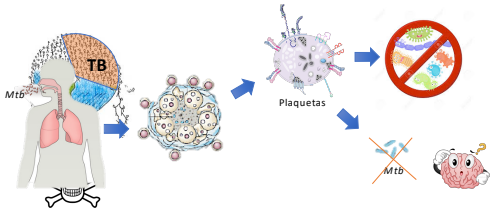
14

Resumen



15

Justificación



16

Objetivo general

Evaluar la respuesta inmune desarrollada por las plaquetas durante la infección con *Mycobacterium tuberculosis*.

17

Hipótesis

Las plaquetas son inmunológicamente activas en la infección con *Mycobacterium tuberculosis*.

18

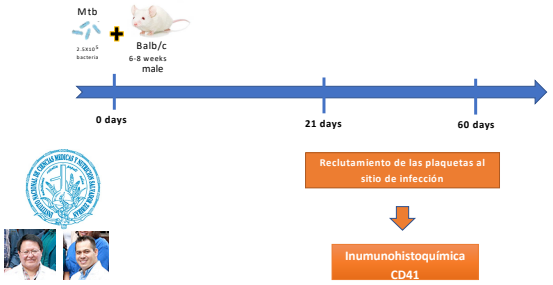
RESULTADOS

19

¿En que momento las plaquetas son reclutadas al sitio de infección?

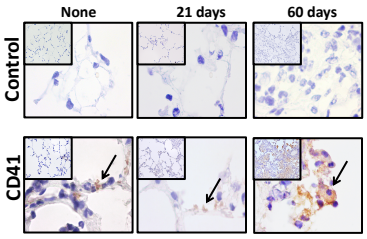
20

ENFOQUE EXPERIMENTAL I: MODELO IN-VIVO



21

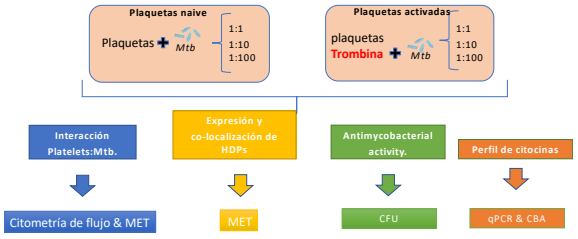
Las plaquetas forman parte de las células residentes en pulmón en tejido sano, aunque presenta un reclutamiento al día 60 post-infección



22

ENFOQUE EXPERIMENTAL II: MODELO IN-VITRO

- Aislamiento de plaquetas humanas a partir de buffy coat (pureza 95%, CD41+).
- Activación de las plaquetas con trombina (0.03 UI/mL) sin afectar la viabilidad celular.



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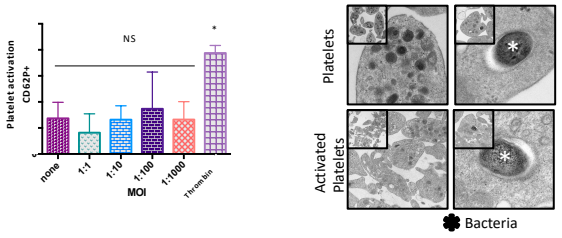
INTERACCIÓN DE PLAQUETAS Y MTB



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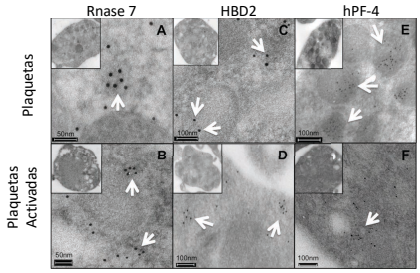
Expresión de HDPs en plaquetas.



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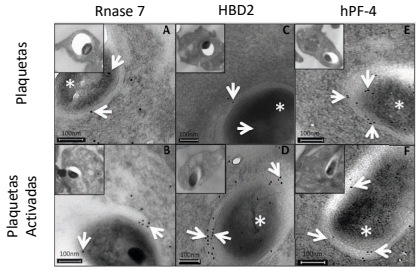
Co-localization de HDPs con Mtb.



¿Pueden eliminar a Mtb?

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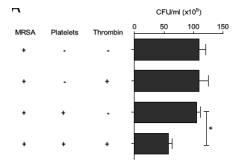
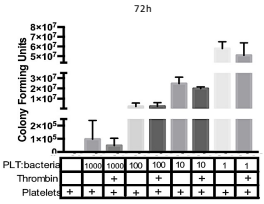
Actividad antimycobacteriana

- Efecto antimicrobiano



Ramadan A. Ali. Journal of Immunology (2016)



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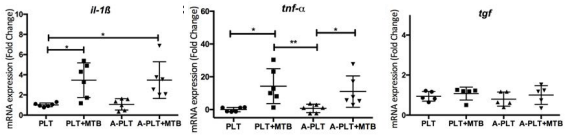
Expresión de citocinas (mRNA)



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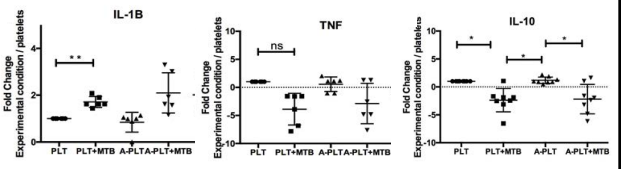
Expresión de citocinas (Proteína)



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Concusiones

- Las plaquetas estan presents en el pulmón como residentes de tejido sano, pero su reclutamiento es inducido al día 60 post-infección.
- La interacción de plaquetas con Mtb, induce la internalización de la misma.
- Mtb induce IL-1ß en las plaquetas (mRNA y proteína) y reduce la liberación of IL-10.
- Plaquetas naïve y activadas expresan HDPs, los cuales co-localizan con Mtb.
- La plaquetas activadas no eliminant la carga de Mtb.

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Perspectivas

- Definir el mecanismo de internalización.
  - Receptores responsables
  - Vías de señalización
- Describir como las plaquetas modulan otros leucocitos durante la infección con Mtb.
  - Macrofagos alveolares: polarización y producción de citocinas.
  - Neutrófilos: NET, migración.
- Definir la función de las plaquetas en tuberculosis.
  - Depleción de plaquetas a especificos puntos del modelo de infección . anti-CD41.
  - Ratón condicional (hPF4-DTR).

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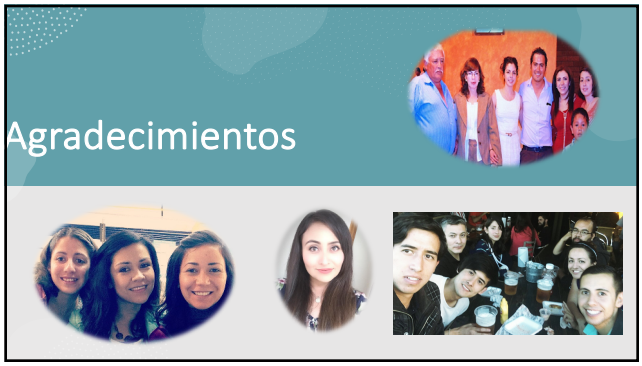

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