Tuberculosis 117 (2019) 36–44

Contents lists available at ScienceDirect

Tuberculosis

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

Mechanisms of Pathogenesis

Interaction of mycobacteria with Plasmin(ogen) affects phagocytosis and granuloma development

Gabriela Echeverría-Valencia[a], Mayra Silva-Miranda[a,d], Euloge Ekaza[b], Antonio J. Vallecillo[a,1], Cristina Parada[a], Isabel Sada-Ovalle[c], Frédéric Altare[b], Clara Espitia[a,*]

[a] Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Apartado Postal 70-228, México D.F, 04510, Mexico
[b] CRCINA, INSERM, Université d'Angers, Université de Nantes, 22 Boulevard Bénoni Goullin, 44200, Nantes, France
[c] Laboratorio de Inmunología Integrativa, Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas, Calz. de Tlalpan, 4502 Del. Tlalpan, Col. Sección XVI, Ciudad de México. C.P, 14080, Mexico
[d] Catedratica CONACyt, Mexico

ARTICLE INFO

Keywords:
Mycobacterium
BCG
Granuloma
Plasmin
Plasminogen
Tuberculosis
Fibrinolytic system

ABSTRACT

Plasminogen and plasmin are fundamental components of the fibrinolytic system that interact with microorganisms generating different immunopathological effects. The molecules of *Mycobacterium tuberculosis* interplaying with plasminogen have already been identified and characterized. In this work, we studied the effects of plasmin(ogen) bound to *Mycobacterium bovis* Calmette-Guérin (BCG) on phagocytosis in THP1 macrophages as well as in granuloma formation and development on *in vitro* human granuloma model. For this purpose, BCG was coated with plasminogen and plasmin, obtained after activation of zymogen by tissue plasminogen activator. The results showed a significant reduction in the number of bacteria phagocytosed by macrophages in presence of plasminogen or plasmin on BCG surface. On the other hand, at 3 days BCG/plasminogen/plasmin induced an increase granuloma numbers with respect to those induced by uncoated bacteria. BCG/plasminogen/environments also showed a significant increase of IL-6 secretion. At 7 days, a reduced number of granulomas and an increased number of bacteria was observed with respect to uncoated BCG environment. Altogether, these results showed that plasmin(ogen) on the mycobacterial surface affects phagocytosis, granuloma development and the cytokine context, thus resulting in an increased number of bacteria in granulomas.

1. Introduction

Tuberculosis (TB) is an infectious disease caused primarily by *Mycobacterium tuberculosis*. This bacterium is regarded as responsible for most of the deaths worldwide caused by a unique infectious agent [1]. In response to this, scientific efforts to control TB have been directed to the knowledge of host pathogen interaction mechanisms and immune response as well as to vaccine design. *M. tuberculosis* mechanisms to invade the host or to establish infection or latent granuloma niche are diverse. After the initial interaction between the pathogen and its host, the molecular dynamic outcome of their interaction contributes to define infection, granuloma development and bacterial survival.

Bacteria-host interactions can be mediated by a plethora of molecules, such as those of the host fibrinolytic system that interact with pathogen proteins. Different bacteria species that bind plasminogen (Plg) have been described, suggesting an important role of these molecules in the host pathogen interplay. Among bacteria that bind Plg/plasmin (Plm) are: *Salmonella enterica* Serovar *typhimurium* and *Escherichia coli* [2], *Steptococcus pneumoniae* [3], *Borreliaburgdorferi* [4], *Leptospira* spp. [5], to mention a few. The outcome of the interaction of bacteria with Plg/Plm can follow different paths. Once Plg is bound to bacterial surfaces, it can be activated to Plm by pathogen proteases or by host plasmin Plg activators (PAs): mammalian tissue Plg activator (tPA) and urokinase Plg activator (uPa). This grants bacteria with a proteolytic activity [6] which pathogens use for invasion and dissemination through tissue barriers. In this process, proteases can cleave molecules of the extracellular matrix (ECM) [7–11] and destruct

* Corresponding author.
E-mail addresses: gfecheverria81@gmail.com (G. Echeverría-Valencia), mayra.silva@iibiomedicas.unam.mx (M. Silva-Miranda), ekazae@yahoo.fr (E. Ekaza), antoniojavierv@yahoo.com (A.J. Vallecillo), cristinaparada2004@yahoo.com.mx (C. Parada), isadamx@gmail.com (I. Sada-Ovalle), frederic.altare@inserm.fr (F. Altare), espitia@biomedicas.unam.mx (C. Espitia).
[1] Present address: Escuela de Medicina Veterinaria y Zootecnia, Facultad de Ciencias Agropecuarias, Universidad de Cuenca, C.P. 010220, Cuenca, Azuay. Ecuador.

https://doi.org/10.1016/j.tube.2019.05.008
Received 23 December 2018; Received in revised form 29 April 2019; Accepted 29 May 2019

immune response related molecules [12,13].

In previous work we demonstrated the existence of Plg receptors (PlgR) in the human pathogen *Mycobacterium tuberculosis* and the presence of putative *M. tuberculosis* PA [14]. Furthermore, the identity of some of *M. tuberculosis* Plg-binding proteins was determined as well as the capacity of Plg bound to mycobacterial receptors to be converted into Plm by the action of the tPA [15].

A role on inflammation had also been described for the molecules of the fibrinolytic system in animals and humans infected with *M. tuberculosis*. For instance, it was observed that the presence of BCG induced upregulation of PAs in animals infected with the bacteria [16,17]. In the other hand, during progressive pulmonary TB in a mouse model, tPA and uPA gradually increased to reach their maximum concentration at the chronic state [18]. The concentration of Plg/Plm inhibitor (PAI) was higher in TB patients in comparison with patients with malignant tumors or liver cirrhosis [19]. In addition, TB patients presented an elevated amount of serum soluble urokinase receptor (suPAR), which has been related to mortality [20]. Likewise, suPAR is considered a biomarker and together with clinical TB score has a mortality prediction value in TB [21]. Furthermore, granuloma development essays using a Plg deficient mouse model showed a Plm effect in the turnover of the extracellular matrix proteins limiting fibrosis, with a restricted effect in early stages of dissemination [22].

In this work, the effects of the interaction of BCG with Plg/Plm were studied. The TB vaccine strain BCG has been used worldwide as an approach model to understand the bacilli pathogenic mechanisms, because of their similarities with the members of the genus and for its relative security during manipulation in comparison with *M. tuberculosis*. The effects of Plg/Plm bound on to the mycobacterial surface (BCG/Plg, BCG/Plm) were investigated at 3 and 7 days of granuloma formation induced *in vitro* by Plg/Plm coated and uncoated BCG. The results showed an upregulated expression of IL-6 secreted at 3 days of granuloma development. In addition, the number of bacteria present in granulomas and granuloma number differed at 3 and 7 days. Interesting, BCG/Plg and BCG/Plm were less phagocytosed by THP1 macrophages than uncoated BCG. Altogether, these results demonstrate that the presence of Plg/Plm on the mycobacterial surface has effects on granuloma development, cytokine production and also in phagocytosis.

2. Materials and methods

2.1. Bacterial culture

BCG and BCG transformed with the gene coding for the green fluorescent protein (GFP) were grown in Middlebrook 7H9 Broth (Difco, Sparks, MD) enriched with 10% ADC (BBL) and supplemented with 0.2% Glycerol and 0.05% Tyloxapol (Sigma, St Louis, MO). Transformed BCG was cultured with 50 μg/mL of Hygromycin (Sigma, St Louis, MO). Bacteria were grown until log phase and then harvested by centrifugation. The pellet was washed 3 times with PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4) and clumps were disaggregated with glass beads. Bacterial suspensions were stored in PBS at −80 °C. Bacterial colony forming units (CFUs) were determined by triplicate, plating serial dilutions of bacteria on Middlebrook 7H11 Agar (Difco, Sparks, MD). Counting the CFUs was performed after incubation at 37 °C for 21 days.

Bacterial pellet obtained as described above was washed with PBS and resuspended in the same buffer placed on ice and sonicated 15 times for 1 min, intercalated with 1 min of pause in a Virsonic 550 sonicator (VirTis). The supernatant obtained correspond to Soluble Extract (SE). Protein concentration was determined by Lowry assay and extract was stored at -70 °C.

2.2. PCR, cloning and expression of annexin A2

Human total RNA was isolated from THP1 cell line using TRIzol (Invitrogen), and cDNA was synthesized using an oligonucleotide d(T) 18 nt and SuperScript™ II reverse transcriptase (Invitrogen) following the manufacturer's specifications. The *ANXA2* coding sequence was amplified with the oligonucleotide probes HAnnA2pETFor 5′-TGTCTA CTGTTCACGAAATCCTGTG-3′ and HAnnA2pETREv 5′–GAT**GCGGCCG CG**TCATCTCCACCACACAGGTAC-3′, which were designed according to the reported sequences (Genebank accession number: NM_001002857.1) (*Not*I site in bold) using the Platinum® *Taq* DNA polymerase (Invitrogen). The PCR product was cloned in the pCR2.1 TOPO TA vector (Invitrogen) the coding sequence was amplified from a positive clone with the high fidelity Platinum® *Pfx* DNA polymerase (Invitrogen) and the same specific oligonucleotides.

The PCR fragment was digested with the *Not*I restriction enzyme (Thermo scientific) and ligated in the modified pET22b (Novagen) expression vector. The vector pET22b had been previously digested with the *Nde*I restriction enzyme (Thermo scientific), blunted with T4 DNA polymerase (Thermo scientific) and finally digested with the *Not*I restriction enzyme (Thermo scientific). Restriction analysis and DNA sequencing confirmed the identity of the fragment cloned into the pET22b-HAnex2 expression vector. All DNA manipulation was made with the *E. coli* TOP10F′ strain (Invitrogen).

E. coli Rosetta (DE3) (Novagen) was transformed with pET22b-HAnex2. The heterologous expression of Annexin A2 was induced in middle log phase using 250 μM IPTG for 5 h. The induced bacterial biomass was collected by centrifuging at 4500 × *g* for 10 min at 4 °C. Bacterial cells were disrupted by sonication in PBS with 2% Triton X-100 and the supernatant was obtained by centrifuging at 12,000 × *g* for 10 min at 4 °C. The human recombinant Annexin A2 (HrAnex2*E.coli*) was purified from the supernatant by using the His Trap HP (GE Healthcare) column and the chromatographer ÄKTA FPLC P-920 (GE Healthcare).

The Ni HisTrap column was washed with distilled water and equilibrated with binding buffer pH 8.0 (150 mM Tris HCl, 50 mM NaCl). The supernatant was added into the column in binding buffer and, the column was washed with washing buffer pH 8.0 (150 mM Tris HCl, 500 mM NaCl, 50 mM Imidazole). Finally, Annexin A2 was eluted applying a linear gradient of Imidazole in elution buffer (150 mM Tris HCl, 50 mM NaCl, 500 mM Imidazole) pH 8.0 until the concentration scoped 0.5 M. The eluted fractions were dialyzed in PBS and analyzed by 12% SDS-PAGE and Western blot.

2.3. Binding of plasminogen to BCG and activation to plasmin by tPA

A total of 200,000 CFU of BCG or BCG/GFP was incubated with 4 μg of Plg (Roche) (final concentration of 1.48 μM) for 1 h at 37 °C with shaking at 200 rpm. Subsequently, samples were centrifuged at 13,000 × *g* for 5 min and pellets were washed with PBS. Plg bound onto BCG surface was activated to Plm, with 50 ng/200,000 CFU of tPA (Calbiochem), for 2 h of incubation at 37 °C and 200 rpm. The pellets were centrifuged and washed as described before. Bacteria were dispersed through syringe needles of 21G, 27G and 30G successively. Finally, bacterial normalization was done by comparing the OD_{600}, with the relative number of bacteria from the original stock.

Activation of Plg to Plm and the proteolytic activity Plm/tPa on BCG surface mesured by an chromogenic activity assay. The bacterial pellets obtained as described above containing BCG, BCG/Plg, BCG/Plm, BCG/tPa, were resuspended in 62.5 μl of PBS and placed on a 96 well microtitration plate (Cliniplate Lab system), then 20 μl of 1.5 mM of Chromozym PL or Chromozym tPa (Roche) was added. The assay was made by duplicate, and activity was measured at OD_{405} nm immediately and then every 60 min, during 17 h at 37 °C in an automatic microtiter plate reader (ThermoLabsystem).

Activation of Plg to Plm on BCG surface was also confirmed by proteolityc cleavage of Annexin A2 used as substrate for Plasmin on BCG Surface 3.5 and 1.75 × 10^4 CFU of BCG were coated with Plg and activated to Plm as described above. Then BCG/Plm (using the relation

of 4 μg Plg/200,000 CFU) were incubated with 0.137 mg/mL Annexin A2 purified from *E. coli*. The assay was performed for 3 h at 37 °C with shaking at 200 rpm. Bacteria were centrifuged, and the supernatant was resolved on a 12% SDS-PAGE, that was subsequently transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk in PBS for 1 h at room temperature (RT). After three washes with PBS-Tween 20 0.05% (PBS-T), membrane was incubated for 1 h at RT with shaking with Anti-His_6-Peroxidase mouse monoclonal antibody (Roche) diluted 1:2000 in PBS with 5% of skim milk. The membrane was washed twice with PBS-T and once with PBS. The buffer was drained and ECL Western Blotting Substrate (Thermo Scientific Pierce) was added following the manufacturer's instructions, to reveal the presence of Annexin A2 and the enzymatic reactions.

In order to search for possible interactions of tPa with BCG proteins, 2 μg of tPa and 10 μg of SE BCG were resolved on a SDS-PAGE, proteins were transferred onto a PVDF membrane, that was blocked as described above. Membrane transferred with SE was incubated with 5 μg of tPA overnight (O.N) at 4 °C. Next day after PBS washes, membranes were incubated with anti-tPA antibody 1:500 (Santa Cruz Sc-5239N-14) by 1 h, at RT. After 5 washes with PBS-T, Protein G-HRP 1:2000 (Invitrogen) in PBS-T BSA was added and membranes were incubated for 1 h more at RT. Reactivity was revealed with 3 mg/mL of 3-3 diaminobenzidine (Sigma) in PBS-T and 30% H_2O_2 diluted 1:1000.

2.4. Cell culture and THP1 infection

Human monocytic leukemia THP1 cells were cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM HEPES, Penicillin (100 U/ml)/Streptomycin (100 μg/mL) and 200 mM Glutamine. For all the experiments, THP1 cells were cultured in 75 cm^2 Falcon culture flasks (Corning) under standard culture conditions (1.0×10^6 cells/ml, 5% CO_2 and 37 °C). Scaling and renewing with fresh media were done every 2–3 days to maintain the culture.

For infection, THP1 cells were differentiated to macrophages using PMA (50 ng/ml, 72 h). The cells were seeded at 1 x 10^5 in 100 μl per well in 96-well plates and cultured at 37 °C, 5% CO_2. Non-adherent cells were carefully removed and the adhered cells were infected with BCG, BCG/Plg or BCG/Plm (MOI 10:1) for 3 h, by using RPMI without both FBS and antibiotics. Subsequently, cells were washed 3 times using RPMI without FBS and Amikacin (200 μg/ml) was added to the wells and the plate was incubated for another hour. Finally, cells were washed and lysed with 100 μl of 0.05% SDS for 10 min. A total of three successive dilutions in base 10 were made and the resulting dilutions were plated on 7H11 medium with Hygromicin. The assay was performed on three occasions, in triplicate each time.

2.5. Granuloma formation and high-content screening technology (HCS) scanning

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors of the Etablissement Français du Sang (Nantes, France). The cells were purified by gradient sedimentation using Histopaque (Sigma Aldrich) or lymphocyte separation medium (PAA laboratory, Les Mureaux, France).

PBMCs were cultured in RPMI 1640 (complete RPMI) (Gibco, Invitrogen) supplemented with 10% of inactivated FBS (Gibco, Invitrogen), 1% Piruvate (Gibco, Invitrogen), 1% non-essential amino acids (Gibco, Invitrogen) and 1% HEPES (Gibco, Invitrogen).

Granuloma formation *in vitro* was performed by incubating PBMCs with BCG-GFP coated with Plg, Plm or with uncoated bacteria at a MOI of 1:150 for 2 h at 37 °C, 200 rpm in RPMI without both FBS and antibiotics. Subsequently, cells were centrifuged and washed twice with PBS 2% FBS and re-suspended in complete RPMI. A total of 3.5 x 10^5 cells/well were inoculated in a 384 well plate. After 3 and 7 days, granulomas obtained from PBMCs were fixed with 4% Paraformaldehyde (Electron Microscopy Sciences) in PBS for 30 min and stained with Hoechst (Sigma) for 30 min. HCS scanning and image analysis were performed as previously described [23]. Briefly, the well plates with the granulomas were fixed, sealed and scanned with CellInsight Thermo Array-Scan HCS equipment (Thermo Fisher Scientific). Observations were performed with a 10× objective and lasers 386 nm and 485 for Hoechst and GFP, respectively. Finally, the images were processed using Cellomics ArrayScan compartmental analysis V3.

2.6. Intracellular cytokine quantification

PBMCs were obtained from healthy donors of the Instituto Nacional de Enfermedades Respiratorias Ismael Cosio Villegas (México DF, México). The cells were separated by gradient sedimentation as described before. A total of 50 x 10^6 PBMCs were seeded in 75 cm^3 cell culture flasks (Corning) and incubated for 1 h. Lymphocytes and non-adherent cells were harvested and kept at 37 °C, whereas infections were performed on the adhered cells at the flask for 2 h with a MOI of 1:150 using BCG, BCG/Plg or BCG/Plm as described before. Non-phagocytosed bacteria were washed using RPMI without FBS and the separated lymphocytes were then added to the flask. The cells were cultured for 3 or 7 days at 37 °C. At these timepoints, granuloma structures were scraped and cells were counted. In addition, culture supernatants obtained by centrifugation were frozen at −80 °C until analysis. Cytokine secretion after harvesting was prevented by incubating the cells with 5 μg/ml Brefeldine A (Sigma Aldrich) for 4 h. Staining was performed using Anti-CD3 Pacific Blue (BD Pharmigen), or Anti-CD163 BV421 (BD Pharmigen); Anti-CD4 Pe/Cy7 (Biolegend) or Anti-CD8 APC/Cy7 (Biolegend). The cells were fixed with 4% of Paraformaldehyde (Sigma Aldrich) in 1% Bovine serum albumin (Sigma Aldrich) PBS for 10 min and washed with PBS. Intracellular cytokine production was quantified by using a solution of 0,1% Saponin (Sigma) in PBS and the following antibodies: Anti–IFN–Gamma APC (BD Pharmigen), Anti-IL-17A PE (BD Pharmigen), Anti-IL-4 APC (BD Pharmigen), Anti- IL-8 PE (BD Pharmigen), Anti- IL-10 APC (BD Pharmigen), Anti- TGF-β1 PE (BD Pharmigen), Anti- MCP-1 PE (BD Pharmigen) and Anti- TNF-α APC (BD Pharmigen). Cytometry was performed using FACS ARIA II and the results were analyzed using FlowJo software.

2.7. Extracellular cytokine granuloma quantification

Cell supernatants obtained from granulomas at 3 and 7 days of development in every analyzed condition, were frozen at −80 °C until quantification. The Human Magnetic Luminex Assay (R&D systems) was used to detect the concentration of cytokines (IFN-γ, IL-4, IL-8, IL-10, IL-17, TNF-α, and IL-6) secreted from granulomas. The platform and software used were MAGPIX (Luminex) according to the manufacturer's instructions.

2.8. Statistical analysis

GraphPad Prism version 6.0c software was used to analyze the results. ANOVA and Tukey analysis was performed to examine the presence of differences between BCG/Plg, BCG/Plm and uncoated BCG. Kruskal-Wallis and Dunn test was performed for the results out of the normality, whereas student-T was used to compare the results between days in the results of number of granulomas and BCG/granuloma.

3. Results

3.1. Binding of plasminogen to BCG and activation to plasmin by tPA

The activation of Plg to Plm on BCG surface in presence of absent of tPA mesured by a chromogenic assay. The results showed that activation of Plg to Plm on BCG surface occurred only in presence of tPA. No

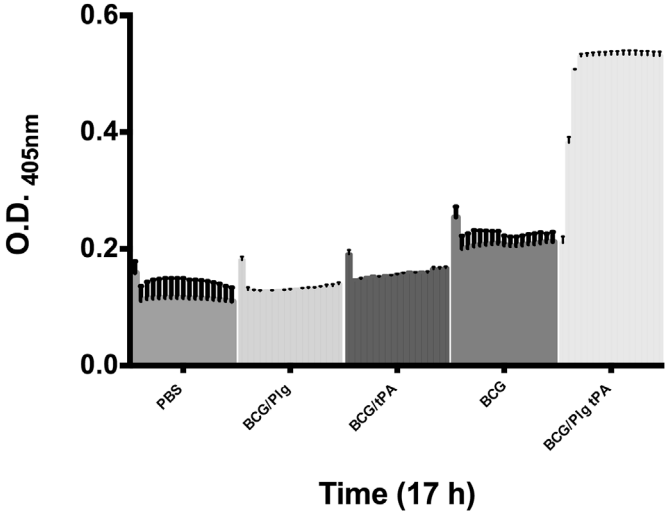

Fig. 1. Chromogenic measuring of Plm and tPA enzymatic activity at the BCG surface. Plm production and activity at the BCG surface was measured using the chromogenic substrate Chromozym PL or Chromozym tPA. Assay was performance at 37 °C and $O.D._{405nm}$ was measured every h for 17 h. Data correspond to a representative experiment from 3 different experiments.

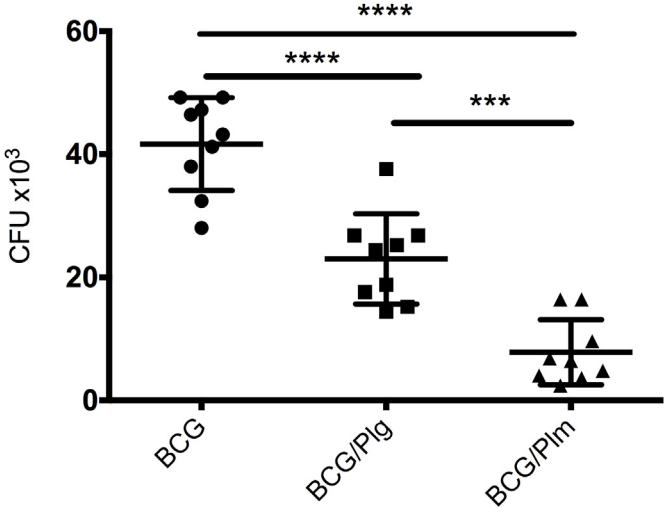

Fig. 3. THP1 infection with BCG/Plg, BCG/Plm and uncoated BCG. Viable bacteria were cultured and counted from THP1 derived macrophages. Data represent three independent experiments; asterisks describe significance ($p < 0,05$).

activation was observed when bacteria were incubated only with Plg or tPA. The activities of Plm and tPA were measured with specific substrates. (Fig. 1).

A ligand blot was also performance to assess whether tPA was able to bind to mycobacteria proteins. No binding of tPa to BCG SE proteins was found. (Supplementary Fig. 1).

On the other hand, the activation of Plg bound to BCG surface to Plm by tPA, was also evaluated by its capacity to cut HrAnex2*E.coli* (Fig. 2). BCG/Plm was able to cleavage HrAnex2*E.coli* and therefore produced a small protein (lane 4), which migrates similarly to the protein produced by the positive control using only Plm (lane 7). Enzymatic activity detection was dependent on the number of BCG/Plm, as seen in lane 3, where no evident reaction was observed with 1.75×10^4 CFU of BCG in contrast when 3.5×10^4 CFU of BCG were used (lane 4). This result demonstrates that Plm on the BCG surface of has an enzymatic activity and can cut the HrAnex2*E.coli*. Indeed, Plm activity occurred only in the bacteria that were coated with Plg and activated by tPA.

3.2. Infection of THP1 cells with BCG coated with Plg/Plm

Macrophages are the main target cell of mycobacterial infection and for this reason; we selected THP1 macrophages to explore the effect of Plg/Plm bound on BCG during phagocytosis. The results showed that Plg or Plm presence on BCG surface reduced the number of bacteria phagocyted by THP1 macrophages. BCG coated with Plg/Plm was significantly less internalized than the uncoated bacteria. However, it is worth of mention that there was a significant reduction of phagocytosis of BCG/Plm with respect to BCG/Plg, suggesting the result that this effect could be due to a possible activation of Plg by host activators. (Fig. 3).

3.3. Analysis of human granulomas using HCS

By HCS scanner, we counted BCG-GFP inside each granuloma structure; we also quantified the granuloma number induced by these bacilli under different conditions. The results obtained demonstrated an increased and significant higher number of granulomas induced by

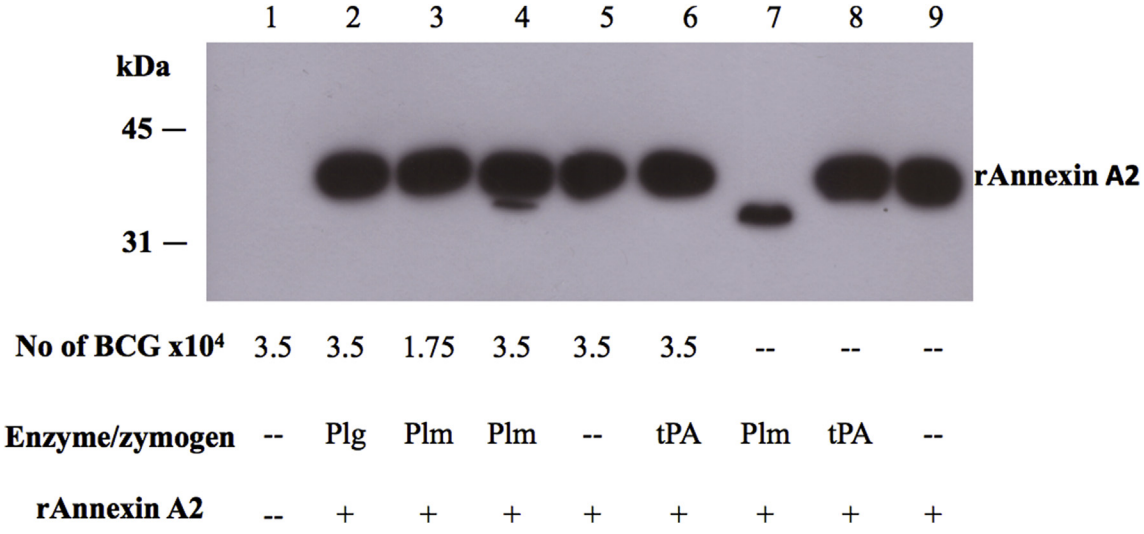

Fig. 2. Analysis of the enzymatic activity of BCG coated with Plg and Plm. The recombinant Annexin A2 exposed to BCG/Plg, BCG/Plm, and uncoated BCG were assessed by Western Blot using an anti His-Peroxidase antibody. Lane 1, uncoated BCG. Lane 2, BGC/Plg-rAnnexin A2. Lanes 3 and 4, BCG/Plm-rAnnexin A2. Lane 5, uncoated BCG -rAnnexin A2. Lane 6, BCG, tPA and rAnnexin A2. Lane 7, rAnnexin A2 cleavage with Plm. Lane 8, tPA-rAnnexin. Lane 9, rAnnexin A2.

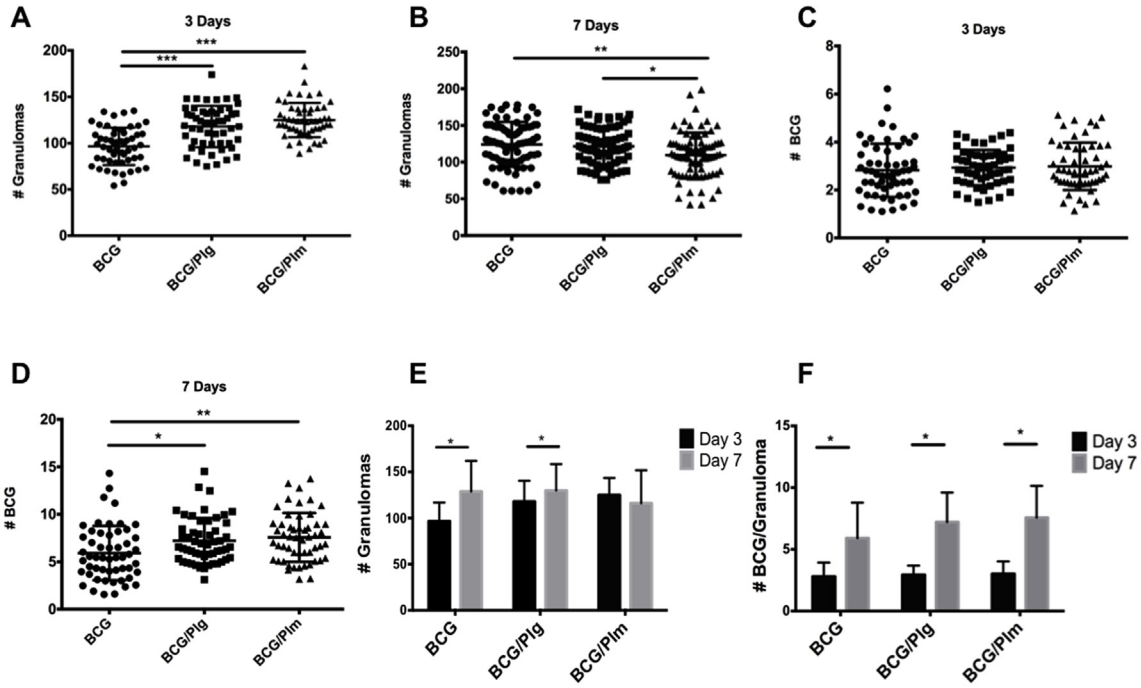

Fig. 4. Dynamics of granuloma formation using BCG/Plg, BCG/Plm, BCG, at 3 and 7 days. The number of granuloma and bacteria within granulomas were quantified using HCS scanner. The results with significant differences between the evaluated conditions are indicated with asterisks. The results represent five independent experiments. **A:** Number of granulomas at 3 days. **B:** Number of granulomas at day 7. **C:** Number of bacteria per granuloma at 3 days. **D:** Number of BCG per granuloma at 7 days. **E:** Comparison of number of granulomas at 3 and 7 days of development. **F:** Comparison of number of BCG per granuloma at day 3 and 7 days.

BCG/Plm. By contrast, BCG/Plg and uncoated BCG induced a reduced number of granulomas (Fig. 4A). At 7 days, the number of BCG/Plm granulomas showed a significant decrease, in comparison to the granulomas induced by uncoated BCG and BCG/Plm (Fig. 4B).

At 3 days of granuloma development, the number of bacteria in each granuloma was apparently similar between the different analyzed conditions, even though the granuloma number was different (Fig. 4C). At 7 days, the number of bacteria inside BCG/Plm granulomas was significantly higher, in contrast with uncoated BCG and BCG/Plg granulomas that showed no change in bacterium content (Fig. 4D).

A comparison between timepoints, demonstrated that BCG and BCG/Plg induced significantly more granulomas at 7 days than at 3 days. By contrast BCG/Plm induced a reduction in granuloma number at 7 days, although the differences were not significant between timepoints (Fig. 4E). The comparison of the bacterial number per granuloma between 3 and 7 days showed a significant rise in all cases at 7 days (Fig. 4F).

3.4. Extracellular cytokine granuloma production

To establish the cytokine environment developed by the *in vitro* granuloma, we quantified by Luminex multiplex assay selected cytokines secreted by these structures at 3 and 7 days of development. At 3 days, BCG/Plm granulomas showed significantly higher concentrations of secreted IL-6 followed by BCG/Plg and BCG granulomas (Fig. 5A). BCG/Plg and BCG/Plm granulomas presented augmented secreted TNF-α, whereas uncoated BCG had a lower amount of this cytokine (Fig. 5B). At 7 days of development, cytokine production was highly variable, BCG induced granulomas displayed higher amounts of secreted IL-8, with respect to the reduced concentration detected in BCG/Plg and BCG/Plm induced granulomas (Fig. 6). Finally, no differences in IL-10, IL-4, IL-17, TNF-α and INF-γ expression was observed between the experimental groups at differences between 3 and 7 days could not be not stablished due to the high variability of the data at 7 days. (Supplementary, Figs. 2 and 3).

3.5. Intracellular cytokine cells quantification

The percentage of CD4, CD8 and CD163 expressing cytokines was quantified at 3 and 7 days of granuloma development. At 3 days, BCG/Plg and BCG/Plm granulomas showed a non-significant reduction of CD4 IL-10 expressing cells, in relation to with uncoated BCG (Fig. 7A). On the other hand at 7 days, CD4 and CD8 TNF-α production was higher, although not significantly, in granulomas induced by BCG/Plg and BCG/Plm (Fig. 7C and D). In addition, the IL-10 CD4 cells CD4 from BCG/Plg and BCG/Plm granulomas showed a decreased trend (Fig. 7B).

4. Discussion

Mycobacterial infection evolution is defined by the formation, containment and resolution of granuloma, all of which depend on the dynamics produced by the bacteria and the host immune response. The granuloma constitutes the fundamental niche where the interaction occurs. Inside this structure, the dynamics can inflect to bacterial growth and survival or bacterial control.

To understand the role that fibrinolytic systems plays in host-bacteria interaction in TB, the granuloma establishment and development was analyzed at days 3 and 7 after in granulomas induced *in vitro* with BCG with BCG surface bound with Plg and Plm generated after activation of Plg by tPA. The effect of Plg/Plm on the bacterial surface during phagocytosis by THP1 macrophages was also evaluated.

The capacity to bind Plg is a conserved characteristic in some bacteria as described earlier. Plg constitutes one of the most important and abundant elements of the fibrinolytic system. It circulates in the blood in a concentration of 180–200 μg/ml [24], and is also located in broncho-alveolar fluid [25]. Moreover, the activation of Plg to Plm is tightly regulated in mammals. In bacteria surface, bound Plg could be activated to Plm by bacteria proteases or by host PAs, turning bacteria

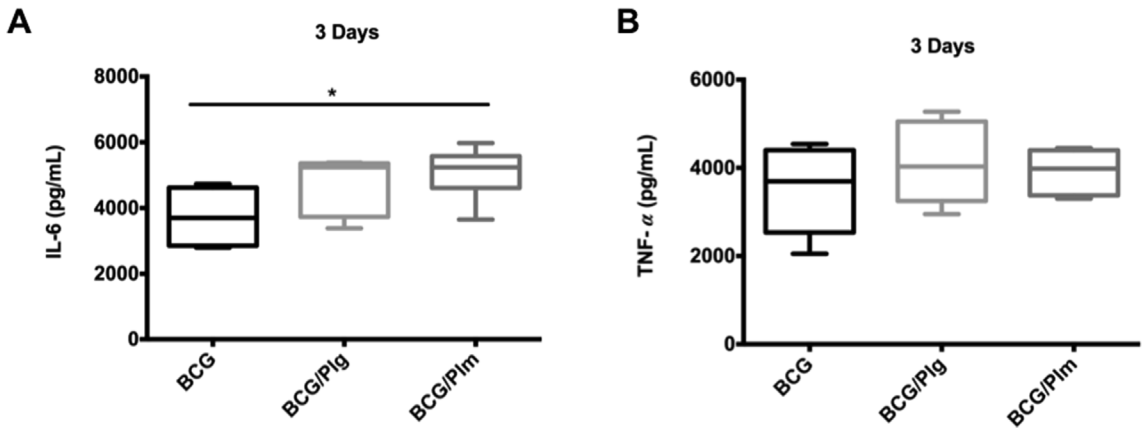

Fig. 5. Extracellular cytokine quantification at 3 days. A: Quantification of IL-6 at the granuloma culture supernatant. **B:** Extracellular TNF-α quantified at 3 days of granuloma formation. The results represent six independent experiments and significant differences are indicated by asterisks.

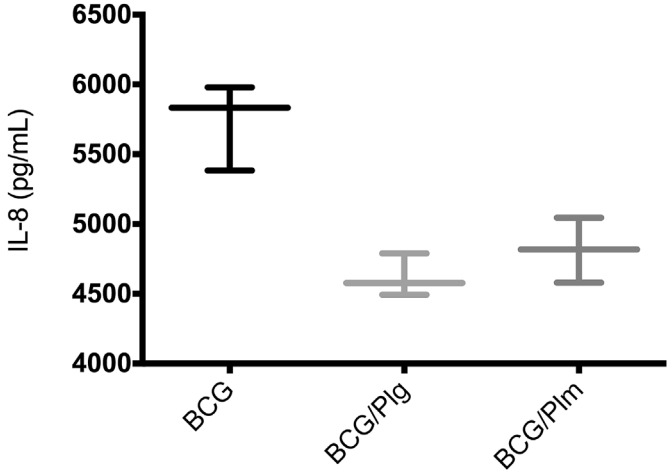

Fig. 6. Extracellular cytokine quantification at 7 days. Extracellular IL-8 was detected 7 days after granuloma formation. The results represent three independent experiments.

into proteolytic organisms with the surface-bound Plm protected against its circulating inhibitor, α2-antiplasmin [6,26,27].

In a previous work, we found that the interaction of *M. tuberculosis* with Plg is mediated by several PlgR, however binding and activation assays carried out with some of those purified recombinant molecules have shown that by their self they were not able to activate Plg in absence of tPA [15,28].

In this work, we demonstrated that Plg bound to live bacteria was activated to Plm only in presence of tPA, in contrast with previous observations of our lab where *M. tuberculosis* SE and whole cell extracts were able to activate Plg bound to fibrin in absence of tPA, suggesting those results the existence of a putative mycobacteria PA [14]. Since, in this study BCG did not expressed the PA, it is possible that the experimental conditions of the activation assay were not favorable for PA expression.

In this way, the intermediate effects of Plg relative to Plm, observed in this study on phagocytosis as well as in granuloma formation and development, despite Plg being in the zymogen form, could be the result of activation of Plg bound to bacteria by host PA.

Phagocytic activity have been described to be affected by Plg, a decreased phagocytosis of apoptotic thymocytes in spleen and peritoneal macrophages of $Plg^{-/-}$ mice, demonstrated the importance of Plg during this process [29]. Although several studies have described the bacterial interactions with elements of the fibrinolytic system during infection in a variety of genus, the effect of Plg/Plm during the mycobacterial phagocytosis or granuloma development remains unclear. Nevertheless, at least two studies [30,31] demonstrated that the presence of Plm on the bacterial surface alters phagocytosis of some pathogenic bacteria. For instance, Plm removes IgG and C3b from the bacterial envelope, thus avoiding *Staphylococcus aureus* neutrophil phagocytosis [30]. Similarly, Plm decreases the uptake of *Francisella tularensis* by macrophages and this reduction is due to the detachment of IgG opsonizing the microorganism [31].

In addition, during mycobacterial infection, the expression of the component molecules of the fibrinolytic system augments in relation to the disease progression, which reaches its maximum at the chronic phase [18]. This increases the possibility of encountering mycobacteria outside the granuloma, with Plg and PA, during advanced TB. The data presented here also showed an increased number of granulomas at day 3 because of BCG/Plm and BCG/Plg. Furthermore, at this stage, the bacteria inside granulomas presented no variation, even when phagocytosis experiments using by THP1 cells demonstrated a reduction of the number of internalized BCG/Plm and BCG/Plg.

Cytokine expression during TB depends on the dynamics of pathogen-cell interaction and controls the development and the progression of the disease. Our results demonstrated differences in the expression of IL-6 at day 3, with higher amounts of this cytokine in the supernatants of BCG/Plg and BCG/Plm granuloma. IL-6 has a pro and anti-inflammatory activity, thus inhibiting virulent mycobacteria growth [32,33]. It also inhibits macrophage response to IFN-γ and avoids bacterial control by preventing IFN-γ -mediated bacterial killing, such as autophagy [34,35]. At day 3, BCG/Plg and BCG/Plm granulomas showed a trend of an increased production of secreted TNF-α. This molecule modulates cell recruitment and granuloma formation through chemokines [36] by playing an important role in the protective response during TB granuloma formation [37]. Defective signaling due to a lack of TNF receptor 1 is related to defective granuloma development [38]. In fact, the administration of neutralizing therapy drugs against TNF-α increases latent TB reactivation rate in patients [39]. In a zebrafish model of *Mycobacterium marinum* infection, the absence of TNF-α produced elevated mortality, bacterial growth, and increased granuloma development that caused necrosis [40]. Thus, the presence of TNF-α, IFN-γ and IL-4 is important to control mycobacterial growth. Whereas IFN-γ alone inhibited the infection, TNF-α and IL-4 did not [41]. Human alveolar macrophages infected with virulent mycobacteria showed an increased expression of TNF-α in comparison with the attenuated strains, furthermore the production of TNF-α in the virulent strains was related to an augmented multiplication of bacteria [42].

Different groups had previously described that Plm inducing increased inflammation in monocytes and in monocyte-derived macrophages. This induction was due to IL-6 and TNF-α expression mediated by signaling after the enzymatic cut of Annexin A2 by Plm [43,44].

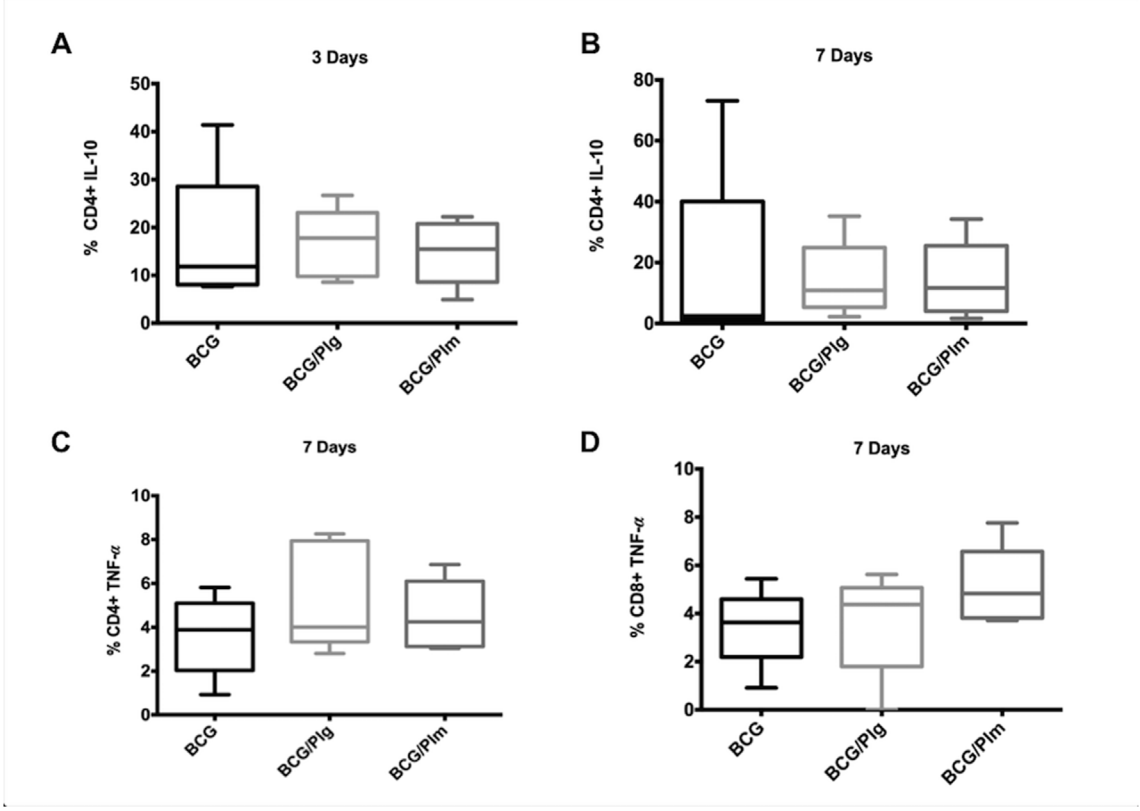

Fig. 7. Intracellular cytokine quantification at 3 and 7 days. A: Quantification through cytometry of the percentage of CD4 cells expressing IL-10 at 3 days. **B:** Quantification of the percentage of CD4 cells expressing IL-10 at 7 days. **C:** Quantification of the percentage of CD4 cells expressing TNF-α at 7 days. **D:** Quantification CD8 expressing TNF-α at 7 days. The results refer to five independent experiments.

Furthermore, in monocytes uPA activated Plm triggered matrix metalloproteinase-1 synthesis through Annexin A2 signaling [45]. Plm activity and Plg presence in macrophages increased LDL catabolism and induced foamy cells [46]. In addition, Plg presence and further activation to Plm regulates cholesterol metabolism and induces CD36. Moreover, Plm stimulated a signaling that produced Leukotriene B4, and that augmented the number of foamy macrophages [47]. Interestingly, during *M. tuberculosis* infection and granuloma development, foamy macrophages had been described as a source of nutrients and a persistence reservoir [48], thus underlining the key role that the activity of Plm is playing in the existence of this reservoir.

At day 3 and 7, intracellular IL-10 in CD4 decreased. IL-10 produced by CD4 cells contributed to minimize the damage produced by the inflammatory response and to generate a balance between bacterial control and latency during *M. tuberculosis* infection [49, 50, 51]. IL-10 an anti-inflammatory cytokine that reduce necrosis [52], attenuated apoptosis in macrophages inoculated by *M. avium* [53] and also inhibited phagosome maturation in macrophages infected with *M. tuberculosis* [54]. IL-10 also blocked the production of TNF-α by macrophages and dendritic cells [55]. However, the lack of IL-10 increased the immune response against *M. tuberculosis,* but also produced an excessive inflammatory behavior in the lungs [56]. IL-10 production in macrophages demonstrated to be interaction-dependent of *M. tuberculosis* Rv1265 with TLR2-ERK [57,58]. In addition, PPE32 interaction with TLR2 upregulated IL-10 by NF-kB and MAPK [59] and Mannose-capped lipoarabinomannan induced IL-10 production by Dectin-2 in antigen-presenting cells [60]. IL-10 production is regulated by the interaction of mycobacterial antigens with TLR and other receptors and mycobacteria covered with Plg or Plm were able to affect the contact with receptors and the resultant cytokine profile. Our results showed a tendency of decreased expression of IL-10 and a trend of increased TNF-α in cells obtained from BCG coated granulomas. This suggests an augmented inflammation context that contributes with IL-6 to produce more granulomas induced by BCG/Plg and Plm at day 3. At day 7 our results showed an increased diversity. Guideon et al. (2015) had previously described the granuloma structure as variable, even in the same host in a macaque model [61]. This group had detected variations in the number of cells, bacteria contents and cytokine expression. Moreover, human and rabbit granulomas analyzed through proteomics and laser microdissection, among other histological and proteomics techniques, showed a physical segregation of the pro and anti-inflammatory signals [62].

At day 7 of granuloma development, BCG/Plg and BCG/Plm granulomas displayed reduced IL-8 secreted. In addition, the number of granulomas developed by coated bacteria was lower and the granulomas presented a higher count of bacteria as compared with BCG. IL-8 is a chemokine related to migration and recruitment [63,64]. Pretreatment with anti-IL-8 in an *in vivo* rabbit model, reduced the presence of neutrophils and granulocytes in the site of tuberculin injection [65]. IL-8 also increased the phagocytic capacity of macrophages and neutrophils, and was responsible for $CD4^+$ and $CD8^+$ recruitment [66]. These findings confirm the importance of IL-8 during granuloma formation and development.

Our work constitutes an original approach to the understanding of the effects of Plg/Plm on mycobacteria in a unique context: the granuloma in TB disease. In summary we detected a reduced phagocytosis of BCG coated by Plg/Plm. During granuloma development, the number of granulomas increased at day 3, until a failure of bacterial control. The cytokine context at day 3 showed an augmented amount of secreted IL-6 in BCG/Plm and BCG/Plg granulomas, which was accompanied by a trend of increased of TNF-α and decreased intracellular IL-10. At day 7, there were an increased number of bacteria perhaps associated with a reduced amount of granulomas in BCG/Plm and BCG/Plg. All this was accompanied by a tendency of increased inflammation by TNF-α CD4

and CD8 cells in the granulomas, and by a reduced IL-8. Finally, the feature adopted by Plg/Plm mycobacterial coating conferred new characteristics inducing changes in the interaction with phagocytes, and even during granuloma development and mycobacterial survival.

Conflicts of interest

The authors declare no conflict of interests.

Acknowledgments

We thank the Institucional Program “Nuevas Alternativas de Tratamiento para Enfermedades Infecciosas” (NUATEI) of the Instituto de Investigaciones Biomédicas UNAM. Gabriela Echeverria-Valencia is a PhD student from the Doctorado en Ciencias Bioquímicas at Universidad Nacional Autónoma de México (UNAM), and has a scholarship from the Consejo Nacional de Ciencia y Tecnología (CONACYT), (CVU/Becario: 378143/252240).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.05.008.

References

[1] World Health Organization. Global tuberculosis report. 2017. p. 2017http://apps.who.int/medicinedocs/documents/s23360en/s23360en.pdf, Accessed date: 3 October 2018.
[2] Kukkonen M, Saarela S, Lähteenmäki K, Hynönen U, Westerlund-Wikström B, Rhen M. Identification of two laminin-binding fimbriae, the type 1 fimbria of *Salmonella enterica* serovar typhimurium and the G fimbria of *Escherichia coli*, as plasminogen receptors. Infect Immun 1998;66:4965–70.
[3] Bergmann S, Rohde M, Chhatwal GS, Hammerschmidt S. α Enolase of *Streptococcus pneumoniae* is a plasmin(ogen) binding protein displayed on the bacterial cell surface. Mol Microbiol 2001;40:1273–87https://doi.org/10.1046/j.1365-2958.2001.02448.x.
[4] Coleman JL, Sellati TJ, Testa JE, Kew RR, Furie MB, Benach JL. *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. Infect Immun 1995;63:2478–84.
[5] Vieira ML, Atzingen MV, Oliveira R, Mendes RS, Domingos RF, Vasconcellos S, et al. Plasminogen binding proteins and plasmin generation on the surface of *Leptospira* spp.: the contribution to the bacteria-host interactions. J Biomed Biotechnol 2012:758513https://doi.org/10.1155/2012/758513.
[6] Pancholi V, Fischetti VA. α-enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic streptococci. J Biol Chem 1998;273:14503–15https://doi.org/10.1074/jbc.273.23.14503.
[7] Fuchs H, Wallich R, Simon MM, Kramer MD. The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. Proc Natl Acad Sci USA 1994;91:12594–8.
[8] Klempner MS, Noring R, Epstein MP, McCloud B, Hu R, Limentani SA, et al. Binding of human plasminogen and urokinase-type plasminogen activator to the Lyme disease spirochete, *Borrelia burgdorferi*. J Infect Dis 1995;171:1258–65https://doi.org/10.1093/infdis/171.5.1258.
[9] Hu LT, Perides G, Noring R, Klempner MS. Binding of human plasminogen to *Borrelia burgdorferi*. Infect Immun 1995;63:3491–6.
[10] Stie J, Fox D. Blood–brain barrier invasion by *Cryptococcus neoformans* is enhanced by functional interactions with plasmin. Microbiology 2012;158:240–58https://doi.org/10.1099/mic.0.051524-0.
[11] Attali C, Durmort C, Vernet T, Di Guilmi AM. The interaction of *Streptococcus pneumoniae* with plasmin mediates transmigration across endothelial and epithelial monolayers by intercellular junction cleavage. Infect Immun 2008;76:5350–6https://doi.org/10.1128/IAI.00184-08.
[12] Singh B, Al-Jubair T, Voraganti C, Andersson T, Mukherjee O, Su YC, et al. *Moraxella catarrhalis* binds plasminogen to evade host innate immunity. Infect Immun 2015;83:3458–69https://doi.org/10.1128/IAI.00310-15.
[13] Chung MC, Tonry JH, Narayanan A, Manes NP, Mackie RS, Gutting B, et al. *Bacillus anthracis* interacts with plasmin(ogen) to Evade C3b-dependent innate immunity. PLoS One 2011;6:e18119https://doi.org/10.1371/journal.pone.0018119.
[14] Monroy V, Amador A, Ruiz B, Espinoza-Cueto P, Xolalpa W, Mancilla R, Espitia C. Binding and activation of human plasminogen by Mycobacterium tuberculosis. Infect Immun 2000;68:4327–30.
[15] Xolalpa W, Vallecillo AJ, Lara M, Mendoza-Hernandez G, Comini M, Spallek R, et al. Identification of novel bacterial plasminogen binding proteins in the human pathogen *Mycobacterium tuberculosis*. Proteomics 2007;7:3332–41https://doi.org/10.1002/pmic.200600876.
[16] Gordon S, Cohn ZA. Bacille Calmette-Guerin infection in the mouse. Regulation of macrophage plasminogen activator by T lymphocytes and specific antigen. J Exp Med 1978;147:1175–88.
[17] Schuyler M, Forman W. Alveolar macrophage plasminogen activator. Exp Lung Res 1984;6:159–69.
[18] Rodríguez-Flores E, Campuzano J, Aguilar D, Hernández-Pando R, Espitia C. The response of the fibrinolytic system to mycobacteria infection. Tuberculosis 2012;92:497–504https://doi.org/10.1016/j.tube.2012.07.002.
[19] Lu XG, Mao JS, Tong JF, Zhu L, Liu J, Gong XB, et al. Fibrinolytic characteristics and their significance in malignant, tuberculous and cirrhotic pleural and ascitic fluids. Int J Lab Hematol 2007;29:132–8https://doi.org/10.1111/j.1751-553X.2006.00835.x.
[20] Eugen-Olsen J, Gustafson P, Sidenius N, Fischer TK, Parner J, Aaby P, et al. The serum level of soluble urokinase receptor is elevated in tuberculosis patients and predicts mortality during treatment: a community study from Guinea-Bissau. Int J Tuberc Lung Dis 2002;6:686–92.
[21] Rudolf F, Wagner AJ, Back FM, Gomes VF, Aaby P, Østergaard L, et al. Tuberculosis case finding and mortality prediction: added value of the clinical TBscore and biomarker suPAR. Int J Tuberc Lung Dis 2017;21:67–72https://doi.org/10.5588/ijtld.16.0404.
[22] Sato J, Schorey J, Ploplis VA, Haalboom E, Krahule L, Castellino FJ. The fibrinolytic system in dissemination and matrix protein deposition during a mycobacterium infection. Am J Pathol 2003;163:517–31https://doi.org/10.1016/S0002-9440(10)63680-2.
[23] Silva-Miranda M, Ekaza E, Breiman A, Asehnoune K, Barros-Aguirre D, Pethe K, et al. High-content screening technology combined with a human granuloma model as a new approach to evaluate the activities of drugs against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2015;59:693–7https://doi.org/10.1128/AAC.03705-14.
[24] Stephens RW, Vaheri A. Plasminogen. In: Kreis T, Vale R, editors. Guidebook to the extracellular matrix and Adhesion proteins. New York: Oxford University Press; 1993. p. 81–2.
[25] Wattiez R, Hermans C, Bernard A, Lesur O, Falmagne P. Human bronchoalveolar lavage fluid: two-dimensional gel electrophoresis, amino acid microsequencing and identification of major proteins. Electrophoresis 1999;20:1634–45https://doi.org/10.1002/(SICI)1522-2683(19990601)20:7<1634::AID-ELPS1634>3.0.CO;2-J.
[26] Lottenberg R, Minning-Wenz D, Boyle MD. Capturing host plasmin(ogen): a common mechanism for invasive pathogens? Trends Microbiol 1994;2:20–4.
[27] de la Paz Santangelo M, Gest PM, Guerin ME, Coinçon M, Pham H, Ryan G, Puckett SE, Spencer JS, Gonzalez-Juarrero M, Daher R, Lenaerts AJ, Schnappinger D, Therisod M, Ehrt S, Sygusch J, Jackson M. Glycolytic and non-glycolytic functions of Mycobacterium tuberculosis fructose-1,6-bisphosphate aldolase, an essential enzyme produced by replicating and non-replicating bacilli. J Biol Chem 2011;46:40219–31http://doi:10.1074/jbc.M111.259440.
[28] de la Paz Santangelo M, Gest PM, Guerin ME, Coinçon M, Pham H, Ryan G, Puckett SE, Spencer JS, Gonzalez-Juarrero M, Daher R, Lenaerts AJ, Schnappinger D, Therisod M, Ehrt S, Sygusch J, Jackson M. Glycolytic and non-glycolytic functions of Mycobacterium tuberculosis fructose-1,6-bisphosphate aldolase, an essential enzyme produced by replicating and non-replicating bacilli. J Biol Chem. 201;46:40219-40231. doi: 10.1074/jbc.M111.259440.
[29] Das R, Ganapathy S, Settle M, Plow EF. Plasminogen promotes macrophage phagocytosis in mice. Blood 2014;124:679–88https://doi.org/10.1182/blood-2014-01-549659.
[30] Rooijakkers SH, Van Wamel WJ, Ruyken M, van Kessel KP, van Strijp JA. Anti-opsonic properties of staphylokinase. Microb Infect 2005;7:476–84https://doi.org/10.1016/j.micinf.2004.12.014.
[31] Crane DD, Warner SL, Bosio CM. A novel role for plasmin- mediated degradation of opsonizing antibody in the evasion of host immunity by virulent, but not attenuated, *Francisella tularensis*. J Immunol 2009;183:4593–600https://doi.org/10.4049/jimmunol.0901655.
[32] Ladel CH, Blum C, Dreher A, Reifenberg K, Kopf M, Kaufmann SH. Lethal tuberculosis in interleukin-6-deficient mutant mice. Infect Immun 1997;65:4843–9.
[33] Leal IS, Smedegard B, Andersen P, Appelberg R. Interleukin-6 and interleukin-12 participate in induction of a type 1 protective T-cell response during vaccination with a tuberculosis subunit vaccine. Infect Immun 1999;67:5747–54.
[34] Dutta RK, Kathania M, Raje M, Majumdar S. IL-6 inhibits IFN-γ induced autophagy in *Mycobacterium tuberculosis* H37Rv infected macrophages. Int J Biochem Cell Biol 2012;44:942–54https://doi.org/10.1016/j.biocel.2012.02.021.
[35] Nagabhushanam V, Solache A, Ting LM, Escaron CJ, Zhang JY, Ernst JD. Innate inhibition of adaptive immunity: *Mycobacterium tuberculosis*-induced IL-6 inhibits macrophage responses to IFN gamma. J Immunol 2003;171:4750–7https://doi.org/10.4049/jimmunol.171.9.4750.
[36] Roach DR, Bean AG, Demangel C, France MP, Briscoe H, Britton WJ. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. J Immunol 2002;168:4620–7https://doi.org/10.4049/jimmunol.168.9.4620.
[37] Kaneko H, Yamada H, Mizuno S, Udagawa T, Kazumi Y, Sekikawa K, et al. Role of tumor necrosis factor-alpha in Mycobacterium-induced granuloma formation in tumor necrosis factor-alpha-deficient mice. Lab Investig 1999;79:379–86.
[38] Bean AG, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD, et al. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. J Immunol 1999;162:3504–11.
[39] Gardam MA, Keystone EC, Menzies R, Manners S, Skamene E, Long R, et al. Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management. Lancet Infect Dis 2003;3:148–55https://doi.org/10.1016/s1473-3099(03)00545-0.
[40] Clay H, Volkman HE, Ramakrishnan L. Tumor necrosis factor signaling mediates

resistance to mycobacteria by inhibiting bacterial growth and macrophage death. Immunity 2008;29:284–94https://doi.org/10.1016/j.immuni.2008.06.011.
[41] Flesch IE, Kaufmann SH. Activation of tuberculostatic macrophage functions by gamma interferon, interleukin-4, and tumor necrosis factor. Infect Immun 1990;58:2675–7.
[42] Engele M, Stössel E, Castiglione K, Schwerdtner N, Wagner M, Bölcskei P, et al. Induction of TNF in human alveolar macrophages as a potential evasion mechanism of virulent *Mycobacterium tuberculosis*. J Immunol 2002;168:1328–37https://doi.org/10.4049/jimmunol.168.3.1328.
[43] Laumonnier Y, Syrovets T, Burysek L, Simmet T. Identification of the annexin A2 heterotetramer as a receptor for the plasmin-induced signaling in human peripheral monocytes. Blood 2007;107:3342–9https://doi.org/10.1182/blood-2005-07-2840.
[44] Li Q, Laumonnier Y, Syrovets T, Simmet T. Plasmin triggers cytokine induction in human monocyte-derived macrophages. Arterioscler Thromb Vasc Biol 2007;27:1383–9https://doi.org/10.1161/ATVBAHA.107.142901.
[45] Zhang Y, Zhou ZH, Bugge TH, Wahl LM. Urokinase-type plasminogen activator stimulation of monocyte matrix metalloproteinase-1 production is mediated by plasmin-dependent signaling through annexin A2 and inhibited by inactive plasmin. J Immunol 2007;179:3297–304https://doi.org/10.4049/jimmunol.179.5.3297.
[46] Haka AS, Grosheva I, Singh RK, Maxfield FR. Plasmin promotes foam cell formation by increasing macrophage catabolism of aggregated low-density lipoprotein. Arterioscler Thromb Vasc Biol 2013;33:1768–78https://doi.org/10.1161/ATVBAHA.112.301109.
[47] Das R, Ganapathy S, Mahabeleshwar GH, Drumm C, Febbraio M, Jain MK, et al. Macrophage gene expression and foam cell formation are regulated by plasminogen. Circulation 2013;127:1209–18https://doi.org/10.1161/CIRCULATIONAHA.112.001214.
[48] Peyron P, Vaubourgeix J, Poquet Y, Levillain F, Botanch C, Bardou F, et al. Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for *M. tuberculosis* persistence. PLoS Pathog 2008;4:e1000204https://doi.org/10.1371/journal.ppat.1000204.
[49] Boussiotis VA, Tsai EY, Yunis EJ, Thim S, Delgado JC, Dascher CC, et al. IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. J Clin Investig 2000;105:1317–25https://doi.org/10.1172/JCI9918.
[50] Gerosa F, Nisii C, Righetti S, Micciolo R, Marchesini M, Cazzadori A, et al. CD4(+) T cell clones producing both interferon-gamma and interleukin-10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients. Clin Immunol 1999;92:224–34https://doi.org/10.1006/clim.1999.4752.
[51] Ranjbar S, Ly N, Thim S, Reynes JM, Goldfeld AE. *Mycobacterium tuberculosis* recall antigens suppress HIV-1 replication in anergic donor cells via CD8+ T cell expansion and increased IL-10 levels. J Immunol 2004;172:1953–9https://doi.org/10.4049/jimmunol.172.3.1953.
[52] Bogdan C, Vodovotz Y, Nathan C. Macrophage deactivation by interleukin 10. J Exp Med 1991;174:1549–55.
[53] Balcewicz-Sablinska MK, Gan H, Remold HG. Interleukin 10 produced by macrophages inoculated with *Mycobacterium avium* attenuates mycobacteria-induced apoptosis by reduction of TNF-a activity. J Infect Dis 1999;180:1230–7https://doi.org/10.1086/315011.
[54] O'Leary S, O'Sullivan MP, Keane J. IL-10 blocks phagosome maturation in *Mycobacterium tuberculosis*-infected human macrophages. Am J Respir Cell Mol Biol 2011;45:172–80https://doi.org/10.1165/rcmb.2010-0319OC.
[55] Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. J Immunol 1991;147:3815–22.
[56] Redford PS, Boonstra A, Read S, Pitt J, Graham C, Stavropoulos E, et al. Enhanced protection to *Mycobacterium tuberculosis* infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung. Eur J Immunol 2010;40:2200–10https://doi.org/10.1002/eji.201040433.
[57] Richardson ET, Shukla S, Sweet DR, Wearsch PA, Tsichlis PN, Boom WH, et al. Toll-like receptor 2-dependent extracellular signal-regulated kinase signaling in *Mycobacterium tuberculosis*-infected macrophages drives anti-inflammatory responses and inhibits Th1 polarization of responding T cells. Infect Immun 2015;83:2242–54https://doi.org/10.1128/IAI.00135-15.
[58] Luo H, Zeng J, Huang Q, Liu M, Abdalla AE, Xie L, et al. *Mycobacterium tuberculosis* Rv1265 promotes mycobacterial intracellular survival and alters cytokine profile of the infected macrophage. J Biomol Struct Dyn 2016;34:585–99https://doi.org/10.1080/07391102.2015.1046935.
[59] Deng W, Li W, Zeng J, Zhao Q, Li C, Zhao Y, et al. *Mycobacterium tuberculosis* PPE family protein Rv1808 manipulates cytokines profile via co-activation of MAPK and NF-κB signaling pathways. Cell Physiol Biochem 2014;33:273–88https://doi.org/10.1159/000356668.
[60] Yonekawa A, Saijo S, Hoshino Y, Miyake Y, Ishikawa E, Suzukawa M, et al. Dectin-2 is a direct receptor for mannose-capped lipoarabinomannan of mycobacteria. Immunity 2014;41:402–13https://doi.org/10.1016/j.immuni.2014.08.005.
[61] Gideon HP, Phuah J, Myers AJ, Bryson BD, Rodgers MA, Coleman MT, et al. Variability in tuberculosis granuloma T cell responses exists, but a balance of pro- and anti-inflammatory cytokines is associated with sterilization. PLoS Pathog 2015;11:e1004603https://doi.org/10.1371/journal.ppat.1004603.
[62] Marakalala MJ, Raju RM, Sharma K, Zhang YJ, Eugenin EA, Prideaux B, et al. Inflammatory signaling in human tuberculosis granulomas is spatially organized. Nat Med 2016;22:531–8https://doi.org/10.1038/nm.4073.
[63] Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M, Ding HA, Gimbrone MA, et al. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature 1999;398:718–23https://doi.org/10.1038/19546.
[64] Mukaida N, Harada A, Matsushima K. Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. Cytokine Growth Factor Rev 1998;9:9–23https://doi.org/10.1016/S1359-6101(97)00022-1.
[65] Larsen CG, Thomsen MK, Gesser B, Thomsen PD, Deleuran BW, Nowak J, et al. The delayed-type hypersensitivity reaction is dependent on IL-8. Inhibition of a tuberculin skin reaction by an anti-IL-8 monoclonal antibody. J Immunol 1995;155:2151–7.
[66] Krupa A, Fol M, Dziadek BR, Kepka E, Wojciechowska D, Brzostek A, et al. Binding of CXCL8/IL-8 to *Mycobacterium tuberculosis* modulates the innate immune response. Mediat Inflamm 2015:24762https://doi.org/10.1155/2015/124762.