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Early Exposure of Human Neutrophils to Mycobacteria Triggers Cell Damage and Pro-Inhibitory Molecules, but not Activation

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1. Introduction

Mycobacterium tuberculosis (Mtb) is responsible for causing tuberculosis (TB) [1], a chronic re-emerging infectious disease with a major impact on public health worldwide [2]. Primary TB occurs by inhalation of microdroplets containing Mtb bacilli [3]. If the innate immune response is not efficient to control and eliminate the mycobacteria, then adaptive T cell responses might contain the bacteria resolving the infection [3-5].

Being TB a long-lasting affliction, most research on the immune responses to TB has addressed the chronic stages. In contrast, the earliest contacts of bacilli with cells responsible of the first defenses have been rather neglected, until recent years. Neutrophils (PMNs) are short-lived leukocytes, functioning as the primary defense against microorganisms [6, 7], with special ability to kill pathogens by phagocytosis, degranulation and the formation of extracellular traps [8-11]. The activation of neutrophils is associated with changes in the spatial and temporal expression of certain molecules [12, 13] such as CD16b, CD11b and CD66b, molecules responsible for adhesion, degranulation and migration, and are therefore well established as neutrophil activation markers [13-19]. On the other hand, it is documented that intracellular pathogens causing chronic infections can co-opt the pathway of a pair of molecules, PD-1 and its ligand PD-L1, involved in decreasing crucial T cell responses, provoking a phenomenon called "T cell exhaustion" [20]. In physiological conditions, this pathway is important for peripheral tolerance and the control of adaptive immune responses [21], but it has been barely explored in TB.

In this work we wanted to assess the phenotype of neutrophils during the very early interactions with three different strains from the *Mycobacterium tuberculosis*-complex.

2. Materials and methods

2.1. Ethics

This research was performed on healthy competent volunteers in accordance with the Declaration of Helsinki of the world Medical Association, and the Mexican General Health Law regarding research. The ethics committee of the National School of Biological Sciences ENCB-IPN approved this study (permission number: "Protocolo #CEI-ENCB 011/2013") and informed written consent was obtained from donors.

2.2. Bacterial strains and cultures

Mycobacterium tuberculosis H37Rv, *Mycobacterium canetti* and *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) strains were grown in Middlebrook 7H9 broth (Difco, Detroit, MI, USA. Cat. 271310), enriched with 10% OADC (Oleic, Albumin, Dextrose, Catalase) (Becton, Dickinson, USA. Cat. 211886) at 37°C until exponential growth phase. Bacteria were diluted in D-MEM (Dulbecco's modified Eagle's medium) (Invitrogen, Grand Island, NY, USA. Cat. 12430) supplemented with 10% FBS (fetal bovine sera) (Invitrogen, Grand Island, NY, USA. Cat. 16000). Mycobacteria were quantified prior to use.

2.3. Isolation of human neutrophils

Human blood neutrophils were isolated from healthy donors using two gradients: Histopaque 1119 (Sigma-Aldrich, St. Louis, MO, USA. Cat. 11191) for 20 min at 800g and Percoll (GE Healthcare Bio-sciences AB, Uppsala, Sweden. Cat. 17-0891-01) at the densities indicated next: 1105 g/mL (85%), 1100 g/mL (80%), 1093 g/mL (75%), 1087 g/mL (70%) y 1081 g/mL (65%), for 20 min at 800g.

2.4. Stimulation and staining of neutrophils

10⁶ healthy neutrophils/mL of RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS) were used either unstimulated or stimulated with 10 nM Phorbol 12-Myristate 13-Acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA. Cat. P-81-39), 5 µg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA. Cat. L-3755), *Mycobacterium bovis* BCG, *Mycobacterium canetti* or *Mycobacterium tuberculosis* H37Rv at 37°C in 5% CO₂ atmosphere for 15, 30 and 60 min. Subsequently, cells were treated 5 min with universal blocking reagent (Biogenex, cat HK085-5K) and stained with different combinations of purified fluorochrome-conjugated antibodies: anti-human CD16b-APC (R&D Systems, Minneapolis, MN, USA. Cat. FAB2546A), CD66b-FITC (AbD Serotec, Kidlington, UK. Cat. MCA216F) and CD11b-PE (BD Pharmingen, San Jose, CA, USA. Cat. 340712). To evaluate the pro-inhibitory phenotype we used anti-human PD-1-FITC (BioLegend, San Diego, CA, USA. Cat. 329903) and anti-human PD-L1-PE (BioLegend, San

Diego, CA, USA. Cat. 329705). After this, cells were washed with 2 mL of FACS solution (PBS 1X, BSA 1%, Sodium azide 0.01%) and the events were acquired in FACS CyAn™ ADP (Beckman Coulter, Inc). FACS data were analyzed using FlowJo X 10.0.7r2 software (Tree Star Inc.)

2.5. Statistics

Statistics were performed with Two-way ANOVA using Bonferroni *t*-test for all multiple pairwise comparisons using GraphPad Prism 5 project (©2013 GraphPad Software).

3. Results

3.1. Neutrophils purity

Blood neutrophils from healthy donors were enriched to about 90% purity, which was checked through staining with Hematoxylin and Eosin (H&E) as well as by flow cytometry parameters such as cell size (FSC) vs. granularity/internal complexity (SSC) (Figure 1 A, B). PMNs were incubated either with culture medium alone, LPS, PMA or the various mycobacteria at two different multiplicity of infection (MOI: 0.1, 1) of *Mycobacterium bovis* BCG, *Mycobacterium canettii* and *Mycobacterium tuberculosis* H37Rv. To corroborate that isolated neutrophils were not contaminated with other leukocyte subpopulations, by means of Fluorescence Activated Cell Sorter (FACS) we evaluated the presence of T lymphocytes (Lc), B Lc and macrophages (Mfs) using specific antibodies to CD3, CD19 and CD14 (Figure 1 B right panel).

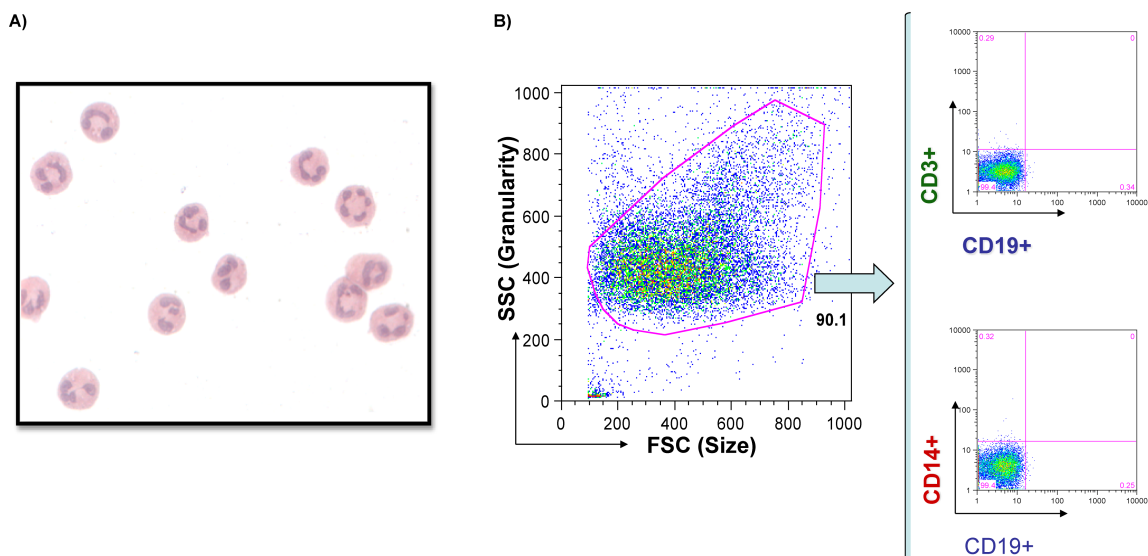


Figure 1. Analysis of peripheral blood neutrophils isolated from healthy donors. Enriched Neutrophils were analyzed both by staining with H&E (A) and by flow cytometry (B). By flow cytometry (B) we evaluated the Size and Granularity (left panel) of the isolated PMNs. The purity of the PMN-enriched cell suspensions was also verified by using markers both for T and B cells (right top panel) and macrophages (right bottom panel).

3.2. *Mycobacterium* affects neutrophils integrity at early times of interactions

By means of flow cytometry we analyzed the neutrophils incubated either with culture medium alone, LPS, PMA, *Mycobacterium bovis* BCG, *Mycobacterium canetti* or *Mycobacterium tuberculosis* H37Rv. We observed an increase in neutrophils autofluorescence, i.e., those that were incubated only with mycobacteria but that were not labeled with any fluorescent reagent at all. Autofluorescence was analyzed in a free channel or filter without staining (FL-6=405 nm) (figure 2). In flow cytometry it has long been described that autofluorescence indicates (membrane) cell damage or even cell death [22-27]. In this case, the neutrophils incubated with BCG or with Mtb H37Rv increased their autofluorescence emission since early times of exposure to mycobacteria. At 15 min of incubation (figure 2B, D) approximately 60-80% of PMNs were autofluorescent, while for neutrophils interacting with *Mycobacterium canetti* the autofluorescence increased approx. 15% at 30 minutes (figure 2G). In contrast, neutrophils incubated with culture medium alone showed basal levels, approximately 10% of autofluorescence (figure 2 A, E, I)

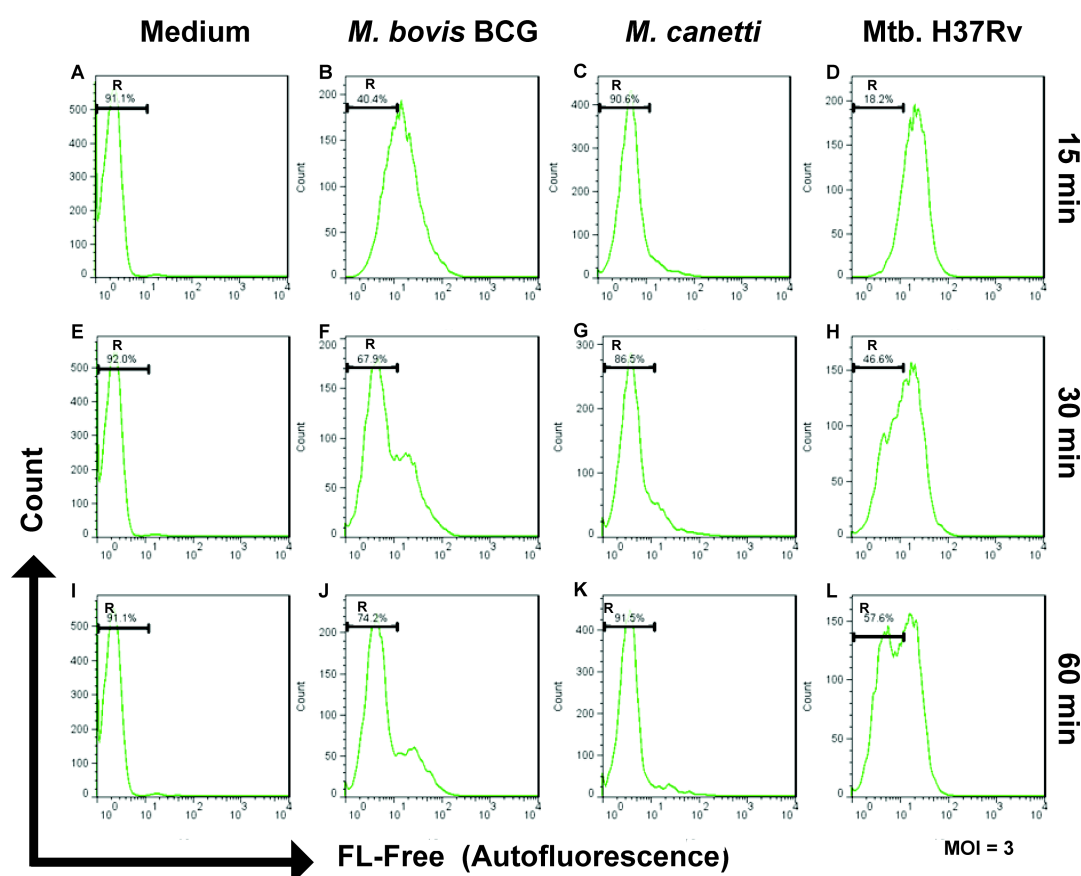


Figure 2. Mycobacteria of different virulence can induce damage to neutrophils at early exposure times, as determined by autofluorescence using flow cytometry. Healthy volunteers' neutrophils were incubated 15, 30 and 60 min with either culture medium alone (Medium), *Mycobacterium bovis* (BCG), *Mycobacterium canetti* or *Mycobacterium tuberculosis* H37Rv. Histograms show the autofluorescence emitted by neutrophils in a free channel (FL-6=405 nm) when using bacilli at a multiplicity of infection (MOI) of 3:1 for each mycobacterial strain.

3.3. Mycobacteria do not activate neutrophils at early times of interaction

Blood neutrophils incubated separately with three different strains of Mycobacteria (BCG, Canetti and Mtb) were evaluated for the expression of CD16b (Figure 3), CD11b and CD66b (Figure 4), at 15, 30 and 60 min incubation. Although these molecules are constitutively expressed on neutrophils, it is well documented that neutrophils modify the expression of these markers upon activation [13, 15, 17, 19, 28-30].

Neutrophils incubated with mycobacteria did not display changes in the percentages of expression of CD16b (Figure 3A), CD11b, CD66b (Figure 4A, C) at the 3 time-points evaluated. There was, however, a decreased in the *median fluorescence intensity* (MFI) for CD16b (figure 3B) and CD66b (figure 4D) and an increment for CD11b (Figure 4B) with Mtb at 15 min incubation, compared with neutrophils in culture medium alone. Short incubation periods with the microbial product LPS or with PMA (both used as positive controls) increased the expression of CD11b (figure 4B) and CD66b (figure 4C), i.e., since 15 min of exposure.

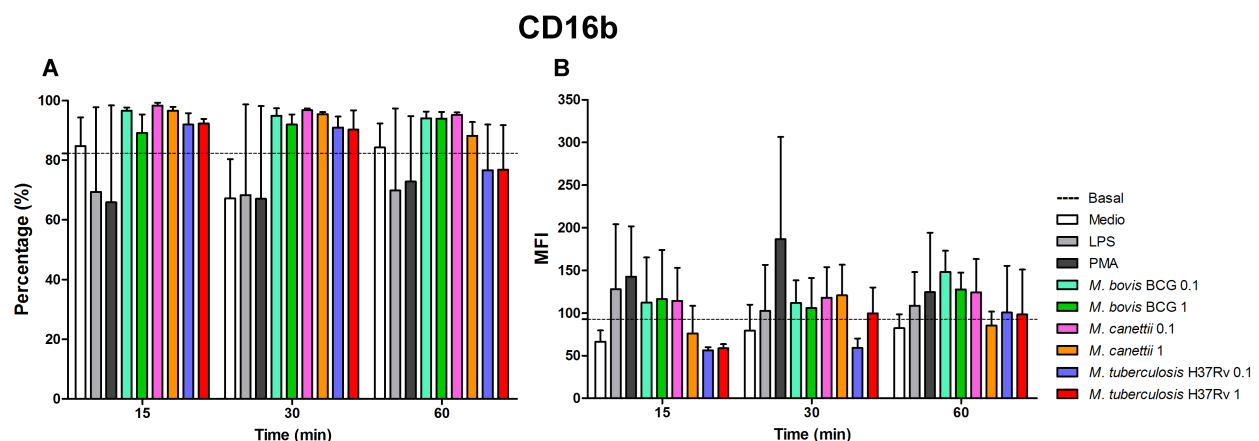


Figure 3. FcγRIIIb-expression in neutrophils exposed to different Mycobacteria. Healthy donor neutrophils were incubated with medium alone (white bars), LPS (gray bars), PMA (black bars), *Mycobacterium bovis* (BCG) MOI 0.1 (aqua bars) and 1 (green bars), *Mycobacterium canetti* MOI 0.1 (pink bars) and 1 (orange bars) and *Mycobacterium tuberculosis* H37Rv MOI 0.1 (blue violet bars) and 1 (red bars). Percentage (A) and MFI (B) of neutrophils labeled for CD16b evaluated at 15, 30 and 60 min incubation with mycobacteria of different virulence. Error bars denote the s.e.m. and dotted lines represent the basal level of CD16b expression (A, B). s.e.m: standard error mean, MFI: Median fluorescence intensity, MOI: Multiplicity of infection, M: *Mycobacterium*.

3.4. Mycobacteria rapidly induce pro-inhibitory molecules on neutrophils

Mycobacteria have evolved diverse mechanisms to escape, divert or even subvert the immune responses [4, 31]. The molecular pair PD1-PD-L1 has been recently shown to induce a phenomenon called T cell exhaustion, and the expression of these two molecules has been shown

manipulated by certain pathogens for their advantage [32, 33]. The percentage of PD1 expression in neutrophils incubated with mycobacteria increased only with Mtb H37Rv (figure 5A, black arrows) since 15 min, compared with basal expression levels. In contrast, PMNs interacting with the other mycobacterial strains did not modify the percentage of PD-1 expression (Figure 5A). Regarding MFI we observed a decrease in PD-1 intensity in neutrophils incubated with Mtb H37Rv MOI:1 at 15 and 30 min, but an increase at 60 min (figure 5B, black arrow). With respect to PD-1 Ligand (PD-L1), the percentage increased in neutrophils incubated with BCG and *Mycobacterium canettii* at 15 min, compared with basal controls (Figure 5C). Neutrophils incubated with Mtb H37Rv show a tendency to increase the MFI of PD-L1 at 15 and 60 min of incubation (figure 5D, blue arrow), this increase was more clear after 60 min, compared with basal intensity and with the other mycobacterial strains tested.

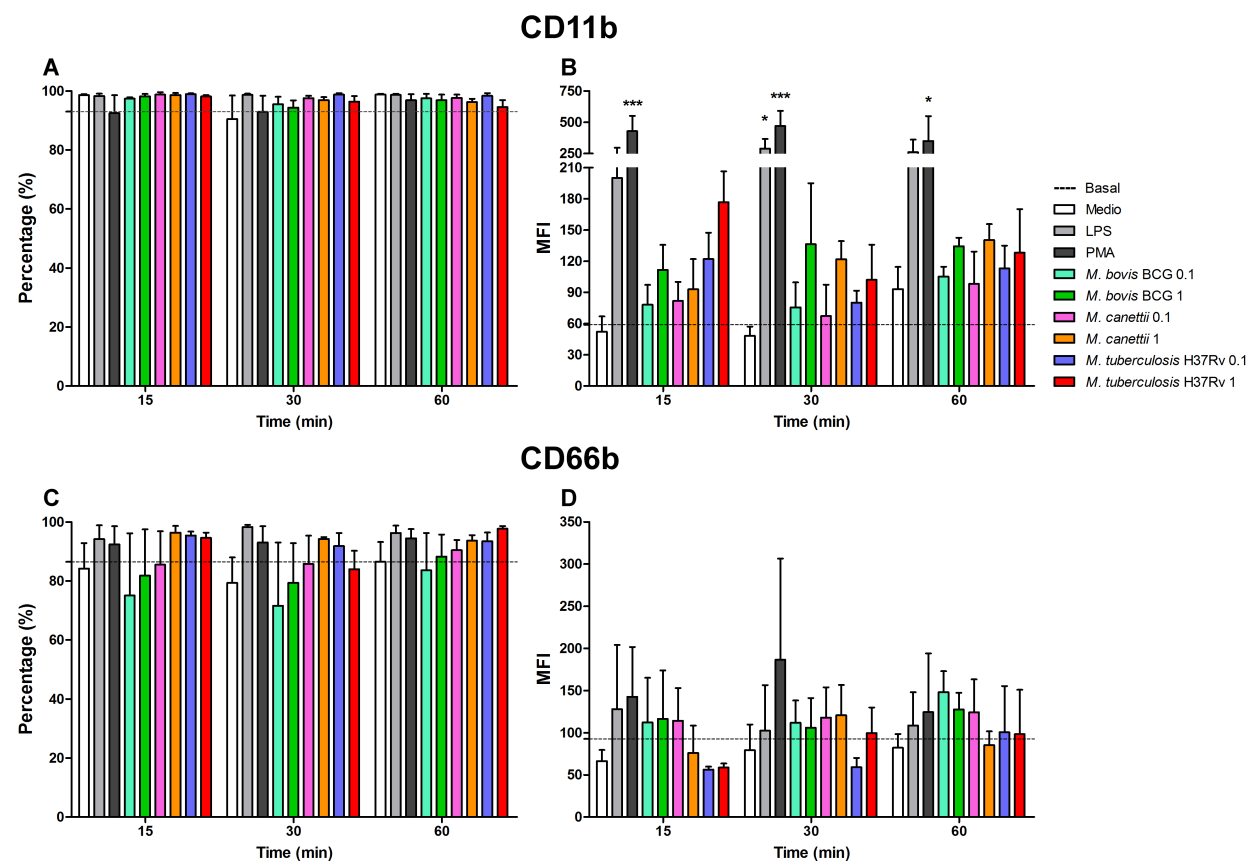


Figure 4. Activation kinetics of human neutrophils exposed to different Mycobacteria. Healthy donor neutrophils were incubated with culture medium alone (white bars), LPS (gray bars), PMA (black bars), *Mycobacterium bovis* (BCG) MOI 0.1 (aqua bars) and 1 (green bars), *Mycobacterium canettii* MOI 0.1 (pink bars) and 1 (orange bars) and *Mycobacterium tuberculosis* H37Rv MOI 0.1 (blue violet bars) and 1 (red bars). Percentage (A, C) and MFI (B, D) of neutrophils labeled for CD11b and CD66b evaluated at 15, 30 and 60 min incubation with different bacilli. Error bars denote the s.e.m. and dotted lines represent CD11b (A,B) and CD66b (C,D) basal expression. * $P=0.01$; *** $P < 0.0001$, one-way ANOVA, s.e.m: standard error mean, MFI: Median fluorescence intensity, MOI: Multiplicity of infection, M: *Mycobacterium*.

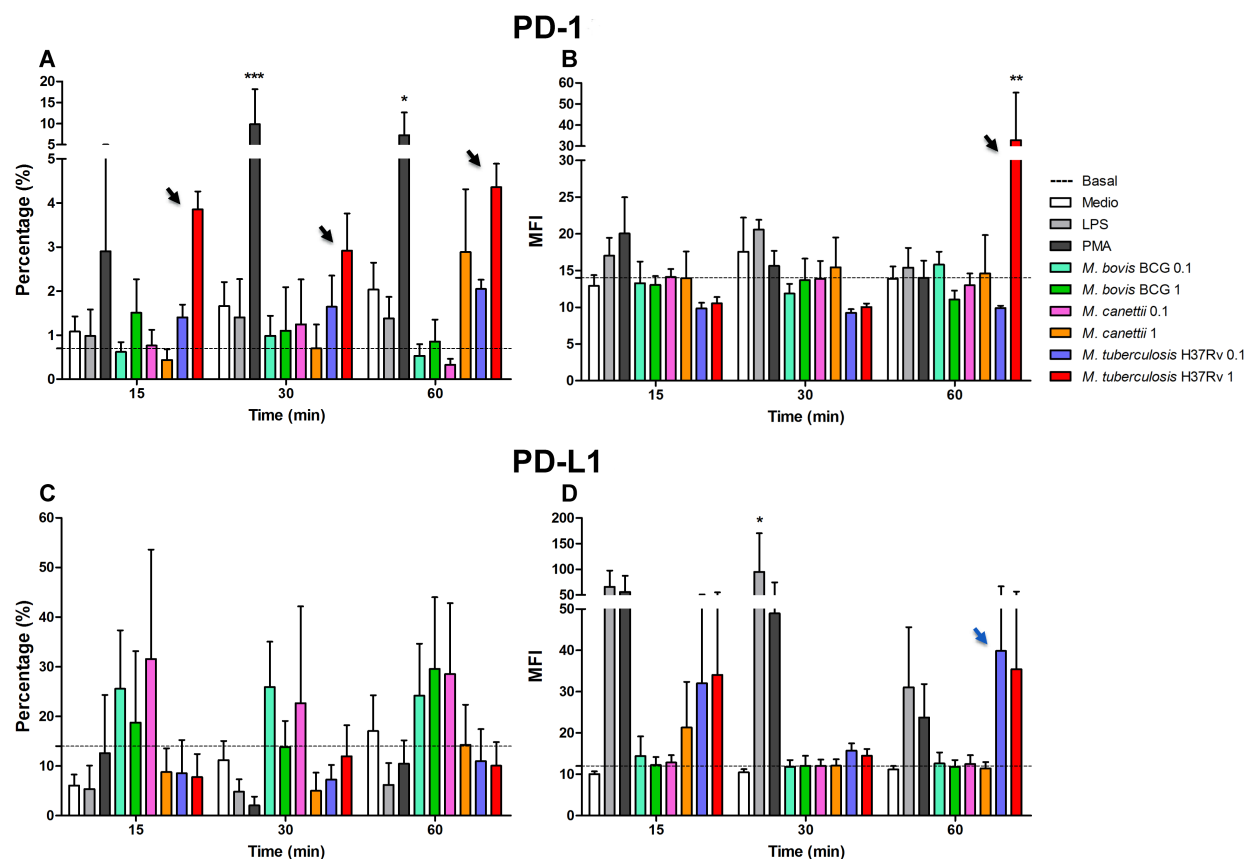


Figure 5. Kinetics of expression of pro-inhibitory molecules in neutrophils exposed to Mycobacteria at early time points. Healthy donor neutrophils were incubated with medium alone (white bars), LPS (gray bars), PMA (black bars), *Mycobacterium bovis* (BCG) MOI 0.1 (aqua bars) and 1 (green bars), *Mycobacterium canettii* MOI 0.1 (pink bars) and 1 (orange bars) and *Mycobacterium tuberculosis* (Mtb) H37Rv MOI 0.1 (blue violet bars) and 1 (red bars). Percentage (A, C) and MFI (B, D) of neutrophils labeled for PD-1 and PD-L1 evaluated at 15, 30 and 60 min incubation with different mycobacteria. Error bars denote the s.e.m. and dotted lines represent the basal levels for PD-1 (A, B) and PD-L1 (C, D). * $P=0.01$; ** $P=0.001$; *** $P < 0.0001$, one-way ANOVA, s.e.m: standard error mean, MFI: Median fluorescence intensity, MOI: Multiplicity of infection, M: *Mycobacterium*.

4. Discussion

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB) [1]. According to the World Health Organization (WHO), TB caused the death of 1.3 million people worldwide in 2012 [2]. Despite the vast amount of TB research in more than 100 years, there are still many aspects of the disease that remain poorly known, for instance the host's innate response during the earliest stages of the infection. The involvement of neutrophils in the immune response against Mtb has been rather neglected until recently. Conceivably, this might be due to the short life span of these cells, the quickness of their mechanisms of action and also to the chronicity of TB.

We aimed at assessing activation as well as pro-inhibitory molecules on neutrophils from healthy donors using flow cytometry, thus PMNs were incubated for short periods with three

different strains of mycobacteria. As positive controls for PMN activation at the time points evaluated, we used two standard components, a microbial one such as lipopolysaccharide (LPS), and a chemical one such as phorbol myristate acetate (PMA). At least two main FACS criteria were evaluated, the percentage of cells and the median fluorescence intensity (MFI). The latter directly reflects the intensity of expression of a given molecule in the cells evaluated.

In flow cytometry the emission of autofluorescence in both animal and plants cells has been indicative of cell damage or even cell death. In addition, the reduction of NADH induces intracellular fluorescence, also indicating cell damage [22-27]. When we cultured mycobacteria with neutrophils, these increased their autofluorescence emission since 15 min, compared with neutrophils in medium alone. Previously, Perskvist *et al.* (2002) demonstrated that Mtb H37Rv induced apoptosis in neutrophils, but this was evaluated at 5h of interaction [34, 35]. However other studies of neutrophils with *Leishmania major* showed increased IL-8 production and inhibition of apoptosis at 24h [36]. Corleis *et al.* (2012) described that at 20 min, neutrophils can kill *Mycobacterium smegmatis* but not *Mycobacterium tuberculosis* [37]. In addition, Ramos-Kichik *et al.* (2009) showed that Mtb induced neutrophil extracellular traps (NETs) at 180 min [38], compared with other microorganisms such as *C. albicans* that do it in shorter time [39].

Few and recent studies have focused on the effects that mycobacteria might have on neutrophils [40-42], for instance whether mycobacteria can induce neutrophil extracellular traps, as seen when PMNs interact with other microorganisms [38]. Of note, even the phenotype of neutrophils during and after their interaction with mycobacteria remains poorly addressed.

From the set of molecules that we measured, some (CD16b, CD11b, CD66b) are well established neutrophil activation markers, while the others (PD1/PD1-L) are very important pro-inhibitory molecules. Interestingly, however, even at the longest time of incubation with mycobacteria (60 min), we did not observe statistically significant differences in the percentages of PMNs expressing CD16b, CD11b or CD66b, compared to neutrophils incubated with the control substances LPS, PMA or with culture medium alone. In contrast, it is known that certain components from microorganisms such as *E. coli*, *S. typhimurium*, *S. flexneri* and *Y. enterocolitica* induce activation of neutrophils at early times (30 min) of exposure [43]. Interestingly, even at 15 min incubation the percentage and the MFI of CD11b, CD66b, CD18 or CD62L increased in human healthy neutrophils incubated with DNA of *E. coli*, LPS, fMLP, C5a and three different strains of *Staphylococcus aureus* [19, 44].

On the other hand, decrease in surface CD16b in neutrophils is also related to the activation of these cells [29, 30]. When we analyzed the median fluorescence intensity (MFI) of CD16b on mycobacteria-incubated neutrophils, we did not find significant differences compared to the controls, although there was a slight decrease at 15 minutes in cells incubated with Mtb H37Rv compared to freshly isolated neutrophils. Regarding the expression of CD11b and CD66b, both LPS and PMA (used as positive controls for stimulation), increased the expression of these two markers since 15 minutes compared to neutrophils in medium alone, indicative of the intact capacity of neutrophils to respond. Although there was a subtle decrease in CD66b expression at 15 minutes of incubation with bacilli, compared to freshly isolated neutrophils.

Previous studies have evaluated the expression of CD16b and CD66b as activation markers in neutrophils from patients with active TB [45]. Likewise, in healthy neutrophils incubated with

clinical isolates of Mtb, the expression of CD16, CD69 and CXCR2 was evaluated, but only at 3 and 18 h [46]. Hilda *et al.* in 2012 demonstrated that the virulent strain Mtb H37Rv can modulate the expression of FcγRII (CD32), FcγRI (CD64), TLR-4 and CXCR3 in neutrophils, while BCG increased CD32 expression only, and a vaccine strain, *Mycobacterium indicus pranii*, did not produce any apparent effect with MOI: 3, all at 4h of incubation [47]. We did not see differences in neutrophil activation in response to the three strains of mycobacteria, regardless of their different virulence.

The PD-1/PD-L1 pathway is important for the establishment and maintenance of peripheral tolerance as well as to modulate immune and inflammatory responses to pathogens [21, 48]. This pathway has been found altered during chronic viral [49, 50], parasitic [51], fungal [52] and bacterial [32] infections, provoking a phenomenon known as “exhaustion” in CD8+T cells, exerting an inhibitory effect related to pathogens evasion of immune responses [20]. Apparently, Mtb H37Rv can also modulate and manipulate the PD/PD-L1 path not only in T cells, but also in antigen presenting cells and even in elements of the innate immune response, such as NK cells [53]. However, this pair of molecules has been poorly explored in neutrophils, especially in the interplay with microorganisms. Therefore, we sought to evaluate also the neutrophils expression of PD-1 and PD-L1 in the early response to mycobacteria. There was an apparent increase in the proportion of neutrophils expressing surface PD-1 following incubation with Mtb H37Rv (MOI 1), which was more evident at 60 minutes; although not statistically significant. These results are interesting and opposed to data by Yao *et al.* (2009) where they reported that neither NK cells, macrophages nor neutrophils express PD-1 during infection with *Listeria monocytogenes* [54]. On the other hand, we did not observe changes in the proportion of neutrophils expressing PD-L1 after incubation with the different mycobacteria strains at the time points we evaluated. Regarding the mean fluorescence intensity (MFI) of PD-1, we observed that at 15 and 30 min there were no differences in its expression in neutrophils incubated with mycobacteria. However, at 60 min the expression of PD-1 increased importantly in neutrophils but only with Mtb H37Rv at MOI 1. When evaluating the MFI for PD-L1 we did not find significant differences among neutrophils incubated with mycobacteria or with culture medium alone. McNab *et al.* (2011) also evaluated these pro-inhibitory molecules but at the mRNA level in the blood from tuberculosis patients [55]. They found that the polymorphonuclear population overexpressed the PD-1 ligand in patients with active TB, in comparison to patients with latent TB and healthy donors.

Our results indicate that, while neutrophils are readily stimulated upon short time of incubation with either LPS or PMA, they do not get activated during early interactions with three mycobacteria of different virulence. This is revealed by the PMNs inability to modify activation molecules which are involved in crucial processes such as migration, adhesion, phagocytosis and degranulation. However, at these early times of interactions with mycobacteria, there are indeed alterations occurring in other important molecules in PMNs, such as PD1/PD1-L.

It is conceivable that early during a mycobacterial infection neutrophils might encounter and interact with other recruited cells of the immune system while still expressing inhibitory molecules such as PD-1 or PD-L1, thus limiting the intensity of the response at the site of infection. The fact that neutrophils infected with Mtb express both the receptor and the ligand

of PD-1 could be related to an autocrine regulation of this population. Further experiments are required to understand the role of the early innate immune response against *Mycobacterium tuberculosis*. Since there are few studies focused on the neutrophils phenotype during the very early interactions with mycobacteria, we consider our results of interest and we expect that this work will instigate more research on the participation of neutrophils during the early stages of mycobacterial infection.

List of abbreviations

BCG: Bacillus Calmette-Guérin

FACS: Fluorescence Activated Cell Sorter

MFI: Median fluorescence intensity

Mfs: Macrophages

MOI: Multiplicity of infection

Mtb: *Mycobacterium tuberculosis*

PMN: Polymorfonuclear leukocytes or Neutrophils

TB: Tuberculosis

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