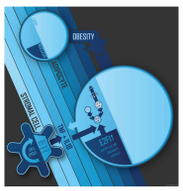
Autophagy



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Neutrophil autophagy during human active tuberculosis is modulated by SLAMF1

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Neutrophils infected with *Mycobacterium tuberculosis* (*Mtb*) predominate in tuberculosis patients' lungs. Neutrophils phagocytose the pathogen, but the mechanism of pathogen elimination is controversial. Macroautophagy/autophagy, a crucial mechanism for several neutrophil functions, can be modulated by immunological mediators. The costimulatory molecule SLAMF1 can act as a microbial sensor in macrophages being also able to interact with autophagy-related proteins. Here, we demonstrate for the first time that human neutrophils express SLAMF1 upon *Mtb*-stimulation. Furthermore, SLAMF1 was found colocalizing with $LC3B^{+}$ vesicles, and activation of SLAMF1 increased neutrophil autophagy induced by *Mtb*. Finally, tuberculosis patients' neutrophils displayed reduced levels of SLAMF1 and lower levels of autophagy against *Mtb* as compared to healthy controls. Altogether, these results indicate that SLAMF1 participates in neutrophil autophagy during active tuberculosis.

Abbreviations. AFB: acid-fast bacilli; BafA1: bafilomycin A_1; CLL: chronic lymphocytic leukemia; DPI: diphenyleneiodonium; EVs: extracellular vesicles; FBS: fetal bovine serum; HD: healthy donors; HR: high responder (tuberculosis patient); IFNG: interferon gamma; IL1B: interleukin 1 beta; IL17A: interleukin 17A; IL8: interleukin 8; LR: low responder (tuberculosis patient); mAb: monoclonal antibody; MAP1LC3/LC3: microtubule associated protein 1 light chain 3; MAPK: mitogen-activated protein kinase; MAPK1/ERK2: mitogen-activated protein kinase 1; MAPK14/p38: mitogen-activated protein kinase 14; *Mtb:* *Mycobacterium* tuberculosis; *Mtb*-Ag: *Mycobacterium tuberculosis*, Strain H37Rv, whole cell lysate; NETs: neutrophils extracellular traps; PPD: purified protein derivative; ROS: reactive oxygen species; PIK3C3/VPS34: phosphatidylinositol 3-kinase catalytic subunit type

3; SLAMF1: signaling lymphocytic activation molecule family member 1; TB: tuberculosis; TLR: toll like receptor.

KEYWORDS: autophagy, immune response, neutrophil, patients, SLAMF1, tuberculosis.

Introduction

Tuberculosis (TB) is the leading cause of death from an infectious microorganism. Actually, *Mycobacterium tuberculosis* (*Mtb*) produces nearly 10 million new cases and 1.5 million deaths per year [1]. The successful establishment of *Mtb* infection depends on its primary interaction with host macrophages, dendritic cells, neutrophils and NK cells [2]. Although great progresses have been made in the characterization of the acquired cellular responses in TB patients, it remains to be elucidated what exactly constitutes a protective response [3]. Furthermore, how *Mtb* is able to evade host immune surveillance and persist, particularly inside phagocytes, remains to be completely understood.

Neutrophils arrive first at the site of infection and are the cells predominantly infected with *Mtb* in patients' lungs [4]. Neutrophils' ability to phagocytose *Mtb* has been demonstrated but its capacity to eliminate the bacteria remains controversial. These cells can sequester *Mtb* in neutrophils extracellular traps (NETs) [5]; cooperate with other cells and release extracellular vesicles (EVs) upon activation. Particularly, EVs charged with *Mtb* activate macrophages, promoting autophagy induction and *Mtb* clearance [6]. However, many studies agree about the fact that neutrophils trigger a hyper-inflammatory response that leads to tissue destruction and mediates damage and lung disease during active TB. In fact, human blood transcriptional profiling performed in TB patients indicated that the TB signature was dominated by a neutrophil-driven interferon (IFN)-inducible gene profile, reflecting the central participation of these cells in active disease [7]. Therefore, a deeper investigation into the biology of neutrophils during *Mtb* infection is crucial for the identification of specific targets to be used in host-directed therapies.

Macroautophagy/autophagy is a central homeostatic mechanism that plays a role in innate and adaptive immunity against intracellular pathogens, including *Mtb* [8]. Furthermore, in macrophages, enhanced autophagy mediates elimination of intracellular *Mtb* through lytic and antimicrobial properties unique to autolysosomes [9]. Additionally, it has been demonstrated that host autophagy coordinate**s** successful antimicrobial responses to mycobacteria during chemotherapy [10]. Moreover, autophagy is essential for major neutrophil functions, including degranulation, reactive oxygen species (ROS) production, and release of NETs [11].

It has also been reported that autophagy can be modulated by different immunological mediators [12]. Accordingly, we have demonstrated that IFNG and IL17A regulate autophagy in *Mtb*-infected monocytes from TB patients in correlation with disease severity [13,14]. SLAMF1 (signaling lymphocytic activation molecule family member 1), a type I glycoprotein from the SLAM subfamily of the CD2-like family of proteins, is a costimulatory molecule involved in host immunity regulation of innate and adaptive responses. We have demonstrated that SLAMF1 contributes to Th1 cytokine responses in human TB [15]. However, SLAMF1 is not only a costimulatory molecule but also a bacterial sensor that helps to remove Gram-negative bacteria by macrophages [16]. Actually, after entering the phagosome by interaction with bacterial outer proteins, SLAMF1 recruits several molecules to the phagosome, including the autophagic protein BECN1/Beclin-1, thus making the connection to the cellular machinery that controls bacterial killing [16,17]. Interestingly, it has been recently shown that SLAMF1 regulates autophagy in B cells from chronic lymphocytic leukemia (CLL) patients, affecting CLL cells responses to autophagy-activating therapeutic agents [18]. However, the expression of SLAMF1 in human neutrophils and its function during autophagy in these cells have not been previously demonstrated. Therefore, to gain insight into the mechanisms that operate during immune responses to *Mtb*, here we

studied the participation of SLAMF1 as a regulator of the autophagy process in neutrophils from TB patients.

Results.

Initially, we analyzed the frequency of neutrophils in peripheral blood from patients with active TB. Our results indicated that the number of neutrophils was significantly higher in patients with severe radiological lesions (bilateral and massive affectation with multiple cavities) as compared to patients with moderate lesions (Fig. 1A). Previously we demonstrated that TB patients could be classified based on *in vitro* lymphocyte responses to *Mtb* antigens. Briefly, high responder (HR) patients are individuals displaying significant proliferative responses, IFNG production and an increased SLAMF1 expression on T lymphocytes upon sonicated *Mtb* antigen (*Mtb*-Ag) stimulation, whereas low responder (LR) patients exhibit low proliferative responses, IFNG production and SLAMF1 expression. Furthermore, LR patients have a more severe pulmonary disease, lower leukocyte counts and a more prolonged illness, compared to HR individuals [15]. Nevertheless, when we examined the number of neutrophils in high and low responder TB patients, similar number of these cells were detected (Fig. 1B). Additionally, we observed a marked increase in neutrophil counts in patients with acid-fast bacilli (AFB)-positive smears (Fig. 1C) as well as a positive correlation between the number of neutrophils and the time of disease evolution (Fig. 1D). Consistent with previous reports [19-21], our findings suggest that increasing numbers of neutrophils might contribute to TB severity. In fact, it was reported that TB cavities contain more neutrophils and less lymphocytes compared to nondestructive pulmonary infiltrates and radiologically unaffected lobes of the lungs [19]. Moreover, we observed that patients undergoing advanced stages of anti-TB treatment displayed lower numbers of neutrophils than those undertaking the first week of chemotherapy (6714 ± 468 neutrophils vs. 5657 ± 710 neutrophils, respectively).

SLAMF1 expression has been demonstrated not only in lymphocytes but in myeloid cells (monocytes and macrophages) [22] as well. However, the expression of SLAMF1 in

human neutrophils has not been evaluated so far. Thus, we initially performed bioinformatic analysis finding evidence of *SLAMF1* RNA expression in unstimulated mature neutrophils from healthy donors (HD) and TB patients (Fig. S1A, B). In line with those initial analyses, in this work, we observed the presence of SLAMF1 in neutrophils from HD and TB patients both by confocal microscopy (Fig. 2A) and flow cytometry (Fig. 2C). Furthermore, after 2 h of *Mtb*-Ag stimulation, we detected an increase in SLAMF1 levels on neutrophils' membrane (Fig. 2B) and an accumulation of localized signal at the intracellular level (Fig. 2A). In fact, Ag challenge significantly elevated the percentage of $SLAMF1^+$ neutrophils as detected by flow cytometry (Fig. 2B-D). Interestingly, upon *Mtb*-Ag stimulation, HD displayed greater percentages of $SLAMF1^+$ neutrophils as compared to TB patients (Fig. 2C and Fig. S2A). Thus, these results show for the first time to our knowledge that SLAMF1 is expressed in human neutrophils and that it increases upon *Mtb*-Ag stimulation. Notably, comparable elevated surface levels of SLAMF1 were detected in both subjects' populations after a robust neutrophil activation with phorbol myristate acetate/PMA (Fig. S2B).

In order to determine the nature of the mycobacterial components involved in the induction of SLAMF1 surface expression on neutrophils, we compared SLAMF1 levels after stimulation with various stimuli. Interestingly, the highest levels of SLAMF1 were induced by compounds that included several proteins, such as purified protein derivative (PPD, Fig. 3A) or culture filtrate proteins (data not shown). Single proteins such as ESAT-6 or CFP-10 did not induce significant amounts of SLAMF1. Moreover, total *Mtb* lipids induced a moderate proportion of $SLAMF1^+$ neutrophils (Fig. 3A). Furthermore, additional antigens with different chemical compositions, such as the lipoglycan ManLam or the 19-kDa *Mtb* lipoglycoprotein (also known as LpqH), both significantly increased the percentage of $SLAMF1^+$ neutrophils as well, in comparison with non-stimulated cells (Fig. 3A). Altogether, our results indicate that diverse bacterial compounds might be recognized by human

neutrophils during *Mtb*-Ag stimulation and induce SLAMF1 surface expression. In particular, compounds including several proteins or lipo/glycans, acted as the predominant stimulators of SLAMF1 expression on human neutrophils.

MAPKs (mitogen-activated protein kinases) have been described to be involved in SLAMF1 expression in lipopolysaccharide (LPS)-stimulated monocytes [23]. Thus, to get insight into the molecular mechanisms that lead to SLAMF1 surface expression on human neutrophils, we next investigated the potential role of the MAPK14/p38 and MAPK1/ERK2-MAPK3/ERK1 pathways. Purified cells were pre-incubated with PD98059 or SB202190 inhibitors for 30 min, and then those neutrophils were stimulated with *Mtb*-Ag for an additional 2 h. Afterwards, SLAMF1 expression was evaluated by flow cytometry. As shown in Figure 3B, the analysis clearly revealed that inhibition of both MAPK/ERK and MAPK14/p38 kinases significantly diminished the percentage of $SLAMF1^+$ neutrophils upon antigen stimulation. Therefore, these findings suggest that these MAPKs participate in the signaling pathway that triggers SLAMF1 surface expression on *Mtb*-Ag-stimulated neutrophils.

Oxidants produced upon NOX (NADPH oxidase) activation play a central role in diverse neutrophil functions [24,25]. Thus, we then analyzed their possible participation in SLAMF1 surface expression. Purified neutrophils were stimulated with or without *Mtb*-Ag in the presence or absence of the NOX inhibitor diphenyleneiodonium (DPI) and, after 2 h, SLAMF1 expression was evaluated using flow cytometry. As shown in Figure 3C, inhibition of reactive oxygen species (ROS) generation significantly diminished the percentage of $SLAMF1^+$ neutrophils. Therefore, our results suggest that expression of SLAMF1 in *Mtb*-Ag-stimulated neutrophils is dependent on ROS production.

Recently, Bologna *et al* reported that reduced SLAMF1 expression in B lymphocytes from CLL patients regulated autophagy affecting drug responses [18].

Additionally, the interaction of SLAMF1 with PIK3C3/VPS34 and BECN1, two proteins involved in the initiation of the autophagy process, had previously been described [16]. In the present work, we observed that *Mtb*-Ag stimulation significantly augmented the percentage of MAP1LC3A/B-II/LC3A/B-II$^+$ neutrophils from TB patients and HD (Fig. 4A), denoting the presence of more autophagosomes by the recognition of mycobacterial antigens. Given that autophagy is a dynamic process and a proportion of autophagosome-anchored-LC3-II is degraded when it reaches the lysosome, the autophagy flux in the presence or absence of bafilomycin A_1 (Baf A1) was analyzed. It is important to notice that the observed increment corresponds to an elevated autophagic flux (Fig. S3A), as showed by a significant increase in the percentage of *Mtb*-Ag-stimulated LC3A/B-II$^+$ neutrophils treated with Baf A1 compared with cells stimulated with *Mtb*-Ag alone. Interestingly, we detected significantly greater autophagy levels in neutrophils from HD as compared to neutrophils from TB patients (Fig. 4A). Moreover, we observed that HR TB patients' neutrophils displayed elevated autophagy levels in comparison to LR TB patients' cells (Fig. S3B).

Considering our data showing expression of SLAMF1 on *Mtb*-stimulated neutrophils, we next evaluated the potential role of this molecule during the autophagy process in neutrophils from TB patients. Isolated neutrophils were stimulated with *Mtb*-Ag and, at the same time, cultured in the presence or absence of an agonistic anti-SLAMF1 antibody. As shown in Figure 4B, we were able to confirm by confocal microscopy an accumulation of localized LC3B foci in *Mtb*-Ag-treated neutrophils. Moreover, activation of SLAMF1 in Ag-stimulated neutrophils significantly increased endogenous LC3B aggregation (Fig. 4B). Furthermore, stimulation with anti-SLAMF1 significantly augmented the percentage of LC3A/B-II$^+$ neutrophils as measured by flow cytometry (Fig. 4C). Importantly, treatment of neutrophils from TB patients with anti-SLAMF1 in the absence of *Mtb*-Ag had no effect on autophagy levels.

We next investigated the autophagy flux in the presence or absence of Baf A1. Anti-SLAMF1 significantly increased LC3B puncta/cell levels in neutrophils from TB patients stimulated with *Mtb*-Ag when Baf A1 was present in the culture (Fig. 4D), suggesting that SLAMF1 activation induces autophagosome formation and a functional autophagy pathway. Furthermore, to support that SLAMF1 regulates autophagy in neutrophils, and because neutrophils are terminally differentiated cells that cannot be transfected, we silenced SLAMF1 by using PLB985 cells, a human myeloid cell line that can be differentiated to a neutrophilic profile. Notably, SLAMF1 silencing markedly reduced the percentage of LC3A/B-II$^+$ cells as compared to PLB985 cells transfected with non-targeting siRNA when stimulated with *Mtb-Ag* (data not shown).

In addition, a positive correlation between the percentage of SLAMF1$^+$ neutrophils and LC3A/B-II$^+$ cells was found (Fig. S4A), further reinforcing the potential role of SLAMF1 during neutrophil autophagy in TB patients. Moreover, we performed a flow cytometry analysis in *Mtb*-Ag-stimulated neutrophils by gating on SLAMF1$^+$ and SLAMF1$^-$ cells. This analysis revealed that autophagy levels detected after *Mtb*-Ag stimulation were mainly observed in SLAMF1$^+$ cells (Fig. S4B). Besides, we observed colocalization of LC3B and SLAMF1 in *Mtb*-Ag-stimulated neutrophils (Fig. 4E). These findings suggest that upon neutrophil activation by *Mtb*-Ag, the triggering of autophagy flux would lead SLAMF1 to LC3$^+$ vesicles.

Considering that ROS are critical intracellular signal transducers sustaining autophagy [26] and our results described above, we next investigated the intracellular levels of ROS upon SLAMF1 activation. As shown in Figure 5A, *Mtb-Ag* triggered ROS production. Moreover, signaling through SLAMF1 in *Mtb*-stimulated neutrophils significantly increased intracellular levels of ROS (Fig. 5A). These results, together with those of Fig. 3C, suggest

that ROS are required for the increase in SLAMF1 membrane expression, and, in turn, SLAMF1 activation promotes ROS production induced by *Mtb*-Ag stimulation.

Because the membrane expression of SLAMF1 induced by *Mtb-Ag* was dependent on ROS production (Fig. 3C) and taking into account that SLAMF1 activation enhanced neutrophil autophagy induced by *Mtb-Ag* (Fig. 4), we then reasoned that DPI should inhibit the increase in autophagy levels upon SLAMF1 stimulation. To test this possibility, HD's neutrophils were treated or not with DPI and then these cells were stimulated with *Mtb*-Ag ± anti-SLAMF1. Finally, autophagy levels were determined. Figure 5B shows that inhibition of ROS generation did not modulate autophagy levels in *Mtb*-Ag-stimulated cells but abrogated the increase in the percentage of LC3A/B-II$^+$ cells observed when neutrophils were stimulated simultaneously with *Mtb-Ag* and anti-SLAMF1. These results, together with those shown in Fig. 4 and Fig. S4A (showing the correlation between SLAMF1 levels and the percentage of LC3A/B-II$^+$ neutrophils), further support the idea that SLAMF1 regulates neutrophil autophagy triggered by *Mtb*.

Discussion.

The outcome of *Mtb* infection depends on the capacity of the host to develop an effective immunity together with its ability to balance the inflammatory responses. Neutrophils participate in granuloma formation but they also mediate tissue destruction and disease severity [27]. These cells appear as multi-functional leukocytes with variable roles in host defense. In fact, the existence of a duality between neutrophils' ability to clear *Mtb* infection and the contribution of increasing numbers of these cells to TB severity and mortality has been documented [20].

SLAMF1 is a self-ligand costimulator that activates lymphocytes. We previously demonstrated that SLAMF1 expression on TB patients' T cells correlates with responsiveness to *Mtb*-Ag [15]. Most members of the SLAM subfamily are expressed on myeloid cells such as macrophages and monocytes [17], but the expression of SLAMF1 in neutrophils has not been reported. In this work we demonstrated that *Mtb*-stimulation significantly induced SLAMF1 expression on human neutrophils. Moreover, we observed that diverse mycobacterial components might be recognized by human neutrophils during stimulation with *Mtb*-Ag and induce SLAMF1 surface expression. In particular, multiple-protein or lipo/glycans compounds were found as the predominant stimulators of SLAMF1 expression (Fig. 3A). These findings are in line with the reported increased SLAMF1 expression on monocytes and macrophages by various toll like receptor (TLR) ligands such as Pam3Cys (TLR1-TLR2), synthetic FSL-1 (TLR2-TLR6), LTA and PGN from *Staphylococcus aureus* (TLR2), flagellin (TLR5) and R848 (TLR7 and TLR8)[23,28]. Indeed, for example, both TLR1-TLR2 and TLR2-TLR6 bind to ManLam [29,30] lipoglycan a major well-characterized *Mtb* virulence factor that regulates the intracellular trafficking network and the immune response of the infected host cell. Nevertheless, recognition by other types of receptors cannot be ruled out.

Additionally, by using MAPK14/p38 and MAP2K/MEK-MAPK/ERK inhibitors we demonstrated that these MAPKs participate in the signaling pathway induced by *Mtb*-Ag that leads to SLAMF1 surface expression on neutrophils (Fig. 3B). Previously, Farina *et al*. have described the participation of MAPK14/p38 signaling during transient SLAMF1 expression on LPS-stimulated monocytes [23]. Besides, Boggaram *et al* reported that ESAT-6, a protein secreted by *Mtb*, induces the expression of IL8 in lung epithelial cells by activating MAPK/ERK and MAPK14/p38 and a rapid induction of ROS production [31]. Moreover, we found that NOX inhibition significantly diminished the percentage of $SLAMF1^+$ neutrophils (Fig. 3C), suggesting that expression of SLAMF1 in response to *Mtb* is dependent on ROS production.

Of further interest was the fact that *Mtb*-Ag-stimulation induced higher numbers of $SLAMF1^+$ neutrophils in HD than in TB patients (Fig. 2C). We have previously reported that *Mtb*-Ag-stimulation induced significantly lower levels of SLAMF1 in T lymphocytes from TB patients as compared to HD [15]. In fact, we observed the highest levels of SLAMF1 in T cells from HD and the lowest levels of the protein in TB patients with the poorest immune response to *Mtb*-Ag (LR TB patients) [15]. Besides, as mentioned previously, Bologna *et al* have reported altered expression of SLAMF1 levels in cells from patients with CLL [18]. In particular, they found that SLAMF1 is lost in CLL cells of patients characterized by a shorter overall survival. Therefore, considering all these previous studies, the reduced levels of SLAMF1 found in neutrophils from TB patients as compared to HD, might be related to imbalances of costimulatory molecules during certain human pathologies [15,18,32-34]. Furthermore, it was suggested that TB disease dramatically alters neutrophils, leading to the accumulation of heterogeneous subpopulations of immature and activated dysfunctional cells [27,35,36], although their occurrence during human TB and the role of SLAMF1 remains to be investigated. Besides, the development of TB is multifactorial, and genetic causes might

influence the pathology and the immune responses against *Mtb* [37-42]. Indeed, single nucleotide polymorphisms may influence the differential SLAMF1 expression on neutrophils from TB patients and HD.

Previous studies showed that SLAMF receptors react with self-proteins, measles virus proteins and bacterial proteins. Additionally, it was shown that SLAMF1 also interacts with the PIK3C3/VPS34-BECN1-UVRAG complex involved in autophagosome maturation [17], and supports phagosome maturation by using the ubiquitous autophagy machinery [16]. Moreover, SLAMF1 was reported to regulate an intracellular autophagic pathway. Actually, SLAMF1 ligation with a monoclonal antibody increases ROS accumulation and induces MAPK14/p38, MAPK8/JNK1-MAPK9/JNK2 and BCL2 phosphorylation, promoting autophagic flux in B cells [18]. However, the role of SLAMF1 as a regulator of autophagy in the context of immunity against *Mtb* has not been described. Here we demonstrated that *Mtb*-Ag induced autophagy in human neutrophils from patients undergoing active TB; and SLAMF1 ligation further increased autophagy in these cells. In fact, a positive correlation between the percentage of surface $SLAMF1^+$ neutrophils and $LC3A/B\text{-}II^+$ cells was found, further reinforcing the potential role of SLAMF1 during neutrophil autophagy in TB patients.

Our findings indicated that the increased SLAMF1 expression induced by *Mtb* involves the MAPKs MAPK14/p38 and MAPK/ERK and NADPH-dependent ROS production. Accordingly, the inhibition of NADPH, by impairing SLAMF1 upregulation, abrogated the increase in autophagy observed upon SLAMF1 ligation in *Mtb*-stimulated cells. These findings, together with the fact that only SLAMF1-expressing neutrophils from TB patients underwent detectable autophagy levels, and the positive correlation found between neutrophil SLAMF1 expression and autophagy levels, support the role of SLAMF1 in regulating neutrophil autophagy upon *Mtb* stimulation. Interestingly, as reported by Bologna *et al* [18] in CLL cells, our findings also showed that SLAMF1 ligation increased ROS

production. If these ROS further modulate SLAMF1 expression at later time points remains to be determined.

Most of the studies on mycobacterium-triggered autophagy were performed in mouse model cell lines or HD primary culture cells, but very limited information exists about autophagy in TB patients. Actually, we showed that *Mtb*-induced IFNG regulates autophagy in TB patients' cells [13]. Consequently, an important aspect of our study is the investigation of autophagy in neutrophils from patients with active TB.

Interestingly, we observed a significant decrease of autophagy levels in neutrophils from TB patients as compared to HD. Because TB patient's displayed lower levels of SLAMF1 as compared to HD, our findings are in agreement with those previously reported in CLL patients, which showed that CLL SLAMF1^{-} cells exhibit an impairment in autophagy activation [18]. Moreover, we further detected a direct correlation between neutrophil numbers and the severity of the disease, as previously demonstrated by other authors [19-21]. In fact, it was reported that TB cavities contain more neutrophils and less lymphocytes compared to nondestructive pulmonary infiltrates and radiological unaffected lobes of the lungs [19]. Hence, considering that autophagy protects against excessive inflammation during *Mtb* infection [43], the reduced autophagy detected in patients' neutrophils might be associated to the common detrimental inflammatory responses occurring during active disease.

Thus, published results [16,18] together with our present findings suggest that SLAMF1 constitutes a human regulator of the autophagy process. Then, if autophagy prevents over-exuberant inflammation, it could be a target for clinical purposes [43]. Therefore, both neutrophil autophagy and SLAMF1 emerge as attractive targets for host-directed therapies either directly increasing the ability of the host to eliminate mycobacteria or limiting collateral tissue damage associated with infection. Because autophagy can

suppress the activation of several inflammasomes by multiple mechanisms but we demonstrated that it also mediates neutrophil IL1B unconventional-secretion [44], further investigations are underway to elucidate the functional consequences of this event.

In conclusion, we identified SLAMF1 as an innate receptor in human neutrophils that can regulate cellular responses against *Mtb*. Therefore, either inducing autophagy in myeloid cells or increasing Th1 responses, SLAMF1 would be a key receptor in human immunity against *Mtb*. Whether SLAMF1 might participate in clinical outcome affecting drug responses of TB patients remains to be elucidated. Altogether, our findings suggest that stimulation of SLAMF1 promotes neutrophil autophagy induced by *Mtb*, suggesting that it might participate in the anti-mycobacterial human response during active TB.

Material and Methods

Subjects.

HIV-uninfected patients with TB were diagnosed at the Servicio de Tisioneumonología Hospital F.J. Muñiz, Buenos Aires, Argentina, based on clinical and radiological data, together with the identification of acid-fast bacilli in sputum. All participating patients had received less than 1 week of anti-TB regular therapy. Table 1 summarizes the demographic and clinical characteristics of enrolled TB patients. Bacillus Calmette-Guerin (BCG)-vaccinated healthy control individuals (HD) from the community participated in this study. Peripheral blood was collected in heparinized tubes from each participant after obtaining informed consent. The local Ethics Committee approved all studies.

Exclusion criteria and patient classification.

All subjects were 18-60 years old and had no history of illnesses that affect the immune system, such as HIV infection, a recent diagnosis of cancer, treatment with immunosuppressive drugs, hepatic or renal disease, pregnancy, or positive serology for other viral (e.g., hepatitis A, B or C), or bacterial (e.g., leprosy, syphilis) infections. Individuals with bleeding disorders or under anticoagulant medication that might be at an increased risk of bleeding during the procedure of obtaining the sample were excluded from the study. Individuals with latent infection were excluded from the present study by using the QuantiFERON-TB® GOLD PLUS kit (Qiagen, 622120 and 622526). TB patients were classified as high-responder (HR) or low responder patients (LR) as previously reported [15], based on *in vitro* lymphocyte responses to sonicated *M. tuberculosis antigen* (*Mtb*-Ag). Briefly, HR patients are individuals displaying significant proliferative responses, IFNG production and an increased SLAMF1 expression against the antigen; whereas LR patients exhibit low proliferative responses, IFNG production and SLAMF1 expression. LR patients

had more severe pulmonary disease, lower leukocyte counts, and a more prolonged illness, compared to HR individuals [15].

Antigen.

In vitro stimulation of cells was performed with a cell lysate from the virulent *Mycobacterium tuberculosis* strain H37Rv, prepared by probe sonication (*Mtb*-Ag) (BEI Resources, NIAID, NIH: *Mycobacterium tuberculosis*, Strain H37Rv, whole cell lysate, NR-14822).

Cell preparations and culture conditions.

Neutrophils were isolated from heparinized blood by centrifugation on Ficoll-Paque (GE Healthcare, 17-1440-03), dextran sedimentation, and hypotonic lysis [45]. Cells were suspended at 2×10^6/mL in RPMI 1640 (Invitrogen, 22400071) supplemented with L-glutamine (2 mM; Sigma Aldrich, G6392), penicillin (100 U/mL; Gibco, 15140122), streptomycin (100 µg/mL; Gibco, 15140122), and 10% fetal bovine serum (FBS; Gibco, 10437028) and used immediately after isolation. Neutrophils were stimulated with *Mtb*-Ag (BEI Resources, NIH, 10 µg/ml) ± anti-SLAMF1 mAb (10 µg/ml, A12; Biolegend, 306310). In some experiments, bafilomycin A_1 (100 nM; Fermentek, 88899-55-2), diphenyleneiodonium chloride (DPI, 100 nM; Sigma Aldrich, 4673-26-1), PD98059 (50 µM; Invivogen, tlrl-pd98) or SB202190 (10 µM; Sigma Aldrich, S7067) were added in different time points of culture.

Purity and viability of neutrophils.

After isolation, neutrophil preparations were stained with an anti-CD14-PE (Biolegend, 325608) antibody and analyzed with a FACS Aria II cytometer (Beckton Dickinson, San Jose, CA, USA) to guarantee that monocyte contamination was <0.5% (Fig. S**5**). Viability was corroborated by staining with propidium iodide (PI; BD, 556547) and flow cytometry analysis.

Flow cytometry.

To determine the level of SLAMF1 membrane expression on neutrophils, cells stimulated with *Mtb*-Ag (10 μg/ml) were blocked in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4)-FBS 5% for 15 min and then stained for surface expression with fluorophore-marked antibodies against SLAMF1 (BD, 559572) before and after culture.

Intracellular staining of endogenous saponin-resistant LC3 was performed as described [46]. Briefly, neutrophils were washed with PBS and then permeabilized with PBS containing 0.05% saponin (Sigma-Aldrich, 47036). In this protocol, the cells are not fixed, therefore LC3-I is washed out of the cell because, unlike LC3-II, it is not anchored to the autophagosome [46]. Cells were then incubated with mouse anti-human LC3A/B antibody (MBL International, M152-3) for 20 min, rinsed with PBS, incubated with anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (eBioscience, 62-6511) for 20 min and rinsed twice with PBS. Negative control samples were incubated with irrelevant isotype-matched monoclonal antibody (Biolegend, 400140). Samples were analyzed on a FACSAria II flow cytometer (BD Biosciences).

Confocal microscopy.

Treated neutrophils (seeded on poly-L-lysine coated coverslips) were fixed and permeabilized (cold methanol, 20 min) and stained with LC3B (Cell Signaling Technology, 2775) and/or SLAMF1 primary antibodies and then with the corresponding secondary antibodies (Alexa Fluor® 488 Goat Anti-Rabbit IgG, Abcam, 150077; Alexa Fluor® 555 Donkey Anti-Mouse IgG, Invitrogen, A31570). Nuclei were counterstained with DAPI. The coverslips were mounted with PBS-mowiol (Sigma-Aldrich, 81381) and imaged using an Olympus FV100 confocal microscope (objective 60/NA1.42, Olympus, Tokyo, Japan).

Image processing.

All the images were processed using ImageJ software (Wayne Rasband, National Institutes of Health). After the image binarization using a defined threshold, the number of LC3 puncta was quantified using the Particle Analyzer plugin. Brightness and contrast were adjusted in all images belonging to the same individual, when needed.

ROS measurement.

Neutrophils from HD were incubated with 2',7'-dichlorofluorescin diacetate (DCFDA, 50 μM; Invitrogen, D399) during 15 min at 37°C. Then, cells were washed and stimulated with or without *Mtb*-Ag (10 μg/ml) ± a SLAMF1 agonist antibody (anti-SLAMF1, 10 μg/ml) for 60 min. Finally, 2',7'-dichlorofluorescin fluorescence was evaluated to monitor ROS production by flow cytometry.

Statistical analysis.

Analysis of variance and post hoc Tukey's multiple comparisons test were used as indicated in the figure legend. Mann-Whitney *U* test and Wilcoxon rank sum test were used for the analysis of unpaired and paired samples respectively. Correlations were calculated using the nonparametric Spearman correlation test. *P* values of < 0.05 were considered statistically significant.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Legends.

Figure 1. Neutrophil counts according to clinical and immunological parameters of tuberculosis patients. Neutrophil numbers per mm^3 in blood from TB patients recruited at Hospital Muñiz, Buenos Aires. Each dot represents the number of neutrophils (per mm^3) for each individual, analyzed according to: (**A**) the severity of the TB disease as determined by the radiological lesions (Severe, n=88; Moderate, n=48; Mild, n=14); (**B**) an immunological classification based on *in vitro* lymphocyte responses to *Mtb*-Ag [15] (HR, High responder, n=65 ; and LR Low responder TB patients, n=97); (**C**) Acid-fast bacilli (AFB) in sputum smear (Ziehl-Neelsen staining; BAAR-, n=13; BAAR+, n=88; BAAR++, n=24; BAAR+++, n=37); (**D**) Time of disease evolution (months with clinical symptoms previous to hospital admission of the individual; one month or less, n=58; more than one month, n=104). Statistical differences were calculated using the nonparametric Mann-Whitney test for unpaired samples. n.s., not significant differences. * $p < 0.05$; ** $p < 0.01$; One-way ANOVA with post hoc Dunnett's multiple comparisons test; Correlation factor (r) and P were calculated using the nonparametric Spearman correlation test.

Figure 2. Expression of SLAMF1 in human neutrophils. Neutrophils from HD and TB patients were stimulated with or without *Mtb*-Ag (10 μg/ml) during different times, and SLAMF1 expression was determined by (**A**) confocal microscopy and (**B, C, D**) flow cytometry. (**A**) Neutrophils from TB patients were stimulated ± *Mtb*-Ag during 2 h. Representative images from ten independent experiments are shown. Scale bars: 5 μm. (**B**) Kinetics of SLAMF1 surface expression as measured by flow cytometry. Each point represents the mean values of the percentage of $SLAMF1^+$ neutrophils ± SEM at 0, 2, 4 or 16 h after *Mtb*-Ag stimulation (n=5). (**C**) SLAMF1 surface expression in neutrophils from HD (n=23) and TB patients (n=18) was determined by flow cytometry. Bar represent mean values

of the percentage of SLAMF1+ neutrophils ± SEM (left axis) and dots represent the number of SLAMF1 neutrophils/10,000 cells (right axis). (**D**) A representative histogram of flow cytometry is shown. Statistical differences were calculated using the Wilcoxon signed rank test for paired samples. * $p < 0.05$, ** $p < 0.01$. # $p < 0.05$; Mann-Whitney nonparametric test for unpaired samples.

Figure 3. Mechanisms of induction of SLAMF1 expression in humans. (**A**) Human purified neutrophils from HD (n=12) donors were stimulated either with *Mtb*-Ag (10 µg/ml), total *Mtb* lipids, purified protein derivative (PPD, 10 µg/ml), ESAT-6 (10 µg/ml), CFP-10 (10 µg/ml), ManLam (10 µg/ml) or 19kDa lipoprotein (10 µg/ml) during 2 h. Finally, SLAMF1 surface expression was evaluated by flow cytometry as previously described. (**B**) Neutrophils from HD (n=7) were pre-incubated with PD98059 (50µM), an MEK1/ERK MAPK inhibitor, or with SB202190 (10µM), a MAPK14/p38 inhibitor, for 30 min. Cells were then stimulated with or without *Mtb*-Ag (10 µg/ml) during 2 h. Afterwards SLAMF1 surface expression was determined by flow cytometry. (**C**) Neutrophils from HD (n=4) were stimulated with or without *Mtb*-Ag (10 µg/ml) in the presence or absence of diphenyleneiodonium (DPI, 10 µM) during 2 h. Afterwards, SLAMF1 surface expression was determined by flow cytometry. (**A, B, C**) Bars represent the mean values of the percentage of SLAMF1+ neutrophils ± SEM. Statistical differences were calculated using one-way ANOVA and post hoc Dunnett multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4. Role of SLAMF1 in neutrophil autophagy. (**A**) Neutrophils from HD (n=12) and TB patients (n=13) were stimulated with or without *Mtb*-Ag (10 µg/ml) during 2 h, and intracellular saponin-resistant LC3A/B-II determination was performed by flow cytometry. (**B, C**) In separate experiments, neutrophils from HD and TB patients were stimulated with or

without *Mtb*-Ag (10 μg/ml) and treated with a SLAMF1 agonist antibody (anti-SLAMF1, 10 μg/ml). Then, autophagy levels in neutrophils were evaluated by (**B**) immunofluorescence against LC3B in neutrophils (n=4) and (**C**) flow cytometry against intracellular saponin-resistant LC3A/B-II (n=16). (**D**) Before autophagy determination, bafilomycin A_1 (Baf A1) (100 nM) was added for the last 60 min of culture. Then, autophagy levels in neutrophils were evaluated by immunofluorescence against LC3B. Bars represent the mean values of LC3 puncta per cell ± SEM. (**E**) Fluorescence distribution of SLAMF1 and LC3B in *Mtb*-Ag-stimulated neutrophils. Neutrophils were fixed after 1 h of Ag stimulation. Then, cells were permeabilized and stained using specific antibodies anti-SLAMF1 (red) and anti-LC3B (green). Images were acquired with a confocal microscope. Representative images of one out of five TB patients are shown. (**A, C**) Bars represent the mean values of the percentage of LC3A/B-II$^+$ neutrophils ± SEM. (**B, D**) Representative images of one experiment are shown. Scale bars: 5 μm. Statistical differences were calculated using one-way ANOVA and post hoc Dunnett multiple comparison test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. # $p < 0.05$; Mann-Whitney nonparametric test for unpaired samples.

Figure 5. SLAMF1 ligation increases ROS levels in human neutrophils. (**A**) Neutrophils from HD (n=8) were incubated with 2',7'-dichlorofluorescein diacetate (DCFDA, 50 μM) during 15 min and then stimulated with or without *Mtb*-Ag (10 μg/ml) ± an SLAMF1 agonist antibody (anti-SLAMF1, 10 μg/ml) during 60 min. Finally, DCFDA fluorescence was evaluated to monitor ROS production by flow cytometry. Left panel: Bars represent the mean values of the mean fluorescence intensity (MFI) of neutrophils of 8 HD ± SEM. Right panel: a representative histogram is shown (Control: non-stained cells). (**B**) Neutrophils from HD (n=5) were stimulated or not with *Mtb*-Ag (10 μg/ml) ± a SLAMF1 agonist antibody (anti-SLAMF1, 10 μg/ml) and treated or not with diphenyleneiodonium (DPI, 10 μM), a NOX

inhibitor, for 2 h. Then, neutrophil autophagy levels were evaluated by intracellular saponin-resistant LC3A/B-II immunostaining and analyzed by flow cytometry. Bars represent the mean values of the percentage of LC3A/B-II^{+} neutrophils ± SEM. No statistical differences were found between *Mtb*-Ag-stimulated neutrophils treated with DPI compared to *Mtb*-Ag-stimulated cells treated with both anti-SLAMF1 and DPI. Statistical differences were calculated using one-way ANOVA and post hoc Dunnett multiple comparison test. * $p < 0.05$ ** $p < 0.01$.

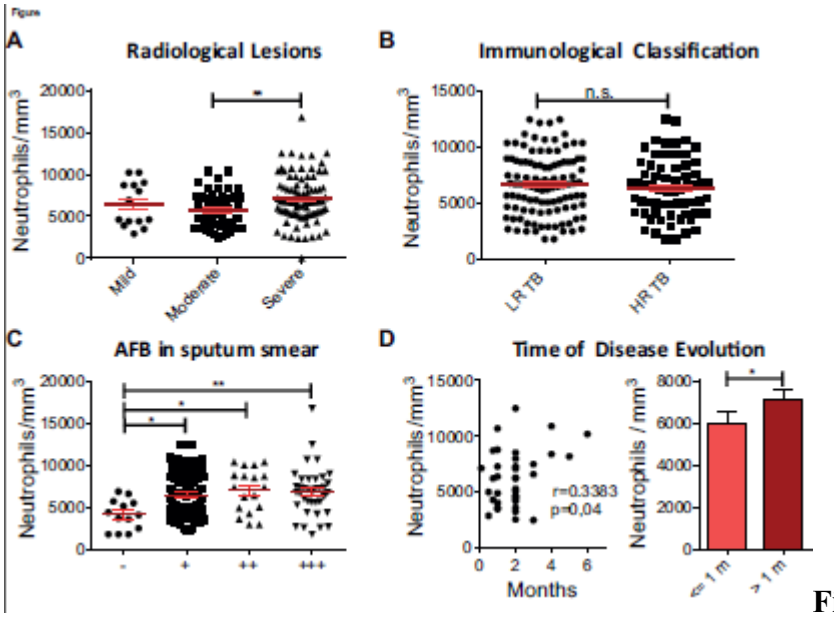

Fig 1

Fig 2

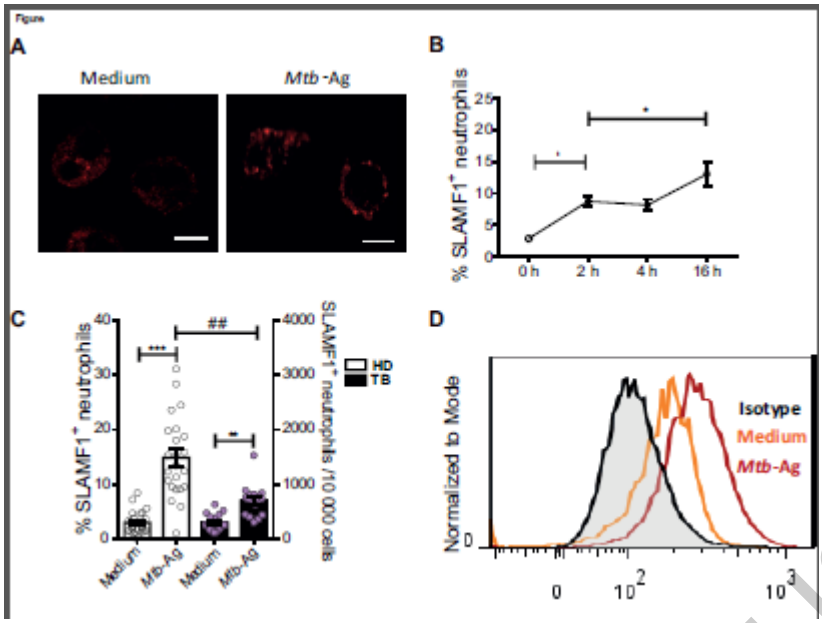

Fig 3

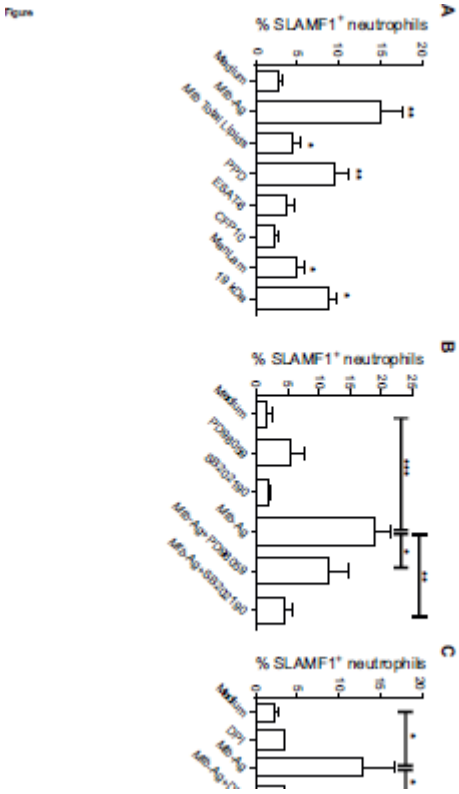

Fig 4