Secretory Leukocyte Protease Inhibitor
A Secreted Pattern Recognition Receptor for Mycobacteria

Sonia A. Gomez[1,2], Claudia L. Argüelles[3], Diego Guerrieri[1], Nancy L. Tateosian[1], Nicolás O. Amiano[1], Rut Slimovich[3], Paulo C. Maffia[1], Eduardo Abbate[4], Rosa M. Musella[4], Verónica E. Garcia[2]*, and H. Eduardo Chuluyan[1]*

[1]Department of Pharmacology, University of Buenos Aires School of Medicine, Buenos Aires, Argentina; [2]Department of Biological Chemistry, University of Buenos Aires School of Sciences, Buenos Aires Argentina; [3]Servicio Derivados de Micobacterias, Instituto Nacional de Producción de Biológicos, Administración Nacional de Laboratorios e Institutos de Salud Dr. Carlos G. Malbrán, Buenos Aires, Argentina; and [4]División de Tisioneumonología, Hospital F. J. Muñiz, Buenos Aires, Argentina

***Rationale*: Human secretory leukocyte protease inhibitor (SLPI) displays bactericidal activity against pathogens such as *Escherichia coli* and *Streptococcus*. Furthermore, it has been reported that murine SLPI shows potent antimycobacterial activity.**

***Objectives*: The aim of the present study was to investigate whether human recombinant SLPI not only kills mycobacteria but also acts as a pattern recognition receptor for the host immune system.**

***Methods*: For the *in vivo* experiment, BALB/c mice were infected by intranasal instillation with *Mycobacterium bovis* BCG and viable BCG load in lung homogenates was later determined. For the *in vitro* experiments, SLPI was incubated overnight with a suspension of *M. bovis* BCG or the virulent strain *Mycobacterium tuberculosis* H37Rv, and the percentage survival as well as the binding of SLPI to mycobacteria was determined. Furthermore, bacteria phagocytosis was also determined by flow cytometry.**

***Measurements and Main Results*: Intranasal SLPI treatment decreased the number of colony-forming units recovered from lung homogenates, indicating that SLPI interfered with *M. bovis* BCG infection. Moreover, SLPI decreased the viability of both *M. bovis* BCG and H37Rv. We demonstrated that SLPI attached to the surface of the mycobacteria by binding to pathogen-associated molecular pattern mannan-capped lipoarabinomannans and phosphatidylinositol mannoside. Furthermore, we found that in the sputum of patients with tuberculosis, mycobacteria were coated with endogenous SLPI. Finally, we showed that phagocytosis of SLPI-coated mycobacteria was faster than that of uncoated bacteria.**

***Conclusions*: The present results demonstrate for the first time that human SLPI kills mycobacteria and is a new pattern recognition receptor for them.**

Keywords: tuberculosis; secretory leukocyte protease inhibitor; phagocytosis

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Human secretory leukocyte protease inhibitor (SLPI) displays bactericidal activity against pathogens such as *Escherichia coli* and *Streptococcus*. Furthermore, it has been reported that murine SLPI shows potent antimycobacterial activity.

What This Study Adds to the Field

Human SLPI binds to *Mycobacterium tuberculosis*, and facilitates phagocytosis and killing of the pathogen.

Tuberculosis, the oldest of the present world's pandemics, remains a substantial worldwide health problem despite the current drug treatments. The disease produced by *Mycobacterium tuberculosis* causes nearly 9 million new cases and 1.7 million deaths annually (1). Moreover, tuberculosis is among the most common causes of morbidity and mortality in patients with HIV (2, 3). The resurgence of tuberculosis and the appearance of multidrug-resistant clinical isolates of *M. tuberculosis* have reaffirmed this disease as a primary public health problem globally. BCG, the only available vaccine, is of variable efficacy, especially in tuberculosis-endemic regions. Thus, to develop a more effective vaccine it is necessary to gain a better understanding of the human immune response to this pathogen.

During the course of *M. tuberculosis* infection, innate immune responses control the spread of the bacteria, but T-lymphocyte recruitment to the lung is later required for containment of the bacteria in the granulomas (4). The fate of an individual exposed to the pathogen will be determined by several factors including genetic background and the strength and specificity of the endogenous defense mechanisms mounted against the pathogen. To control the spread of bacteria, the innate immune system of the host detects conserved molecular structures, called pathogen-associated molecular patterns (PAMPs), produced exclusively by microorganisms and that are essential for the physiology of the microbes (5). PAMPs are recognized by germline-encoded pattern recognition receptors (PRRs) of the host, some of them (e.g., Toll-like receptor-2 [TLR2] and TLR4) present on the surface of cells from the innate immune system. In contrast, other PRRs, such as TLR7, are found inside the cells. Finally, the last group of known PRRs, such as surfactants and C-reactive protein, are soluble and present in extracellular body fluids. The recognition of PAMPs by PRRs facilitates uptake of the pathogen or the signaling needed for induction of the appropriate immune response of the host (5, 6). Virulent *M. tuberculosis* and *Mycobacterium bovis* BCG display different PAMPs, such as lipoproteins (19 kD) and glycoproteins (mannosylated lipoarabinomannan [ManLAM] and phosphatidylinositol mannoside [PIM]) (7) present on the cell envelope of the bacteria. These mycobacterial PAMPs are detected by different PRRs, such as mannose receptor, complement receptors, TLR2, and TLR4 (8).

(*Received in original form April 25, 2008; accepted in final form November 13, 2008*)

* These authors contributed equally to this work.

Supported by a grant from the Universidad de Buenos Aires (UBACYT2004-2007, M019; UBACYT2008-2010, M011) and Agencia (BID 1728/OC-AR PICT 15069, 1476, and 25996).

Correspondence and requests for reprints should be addressed to H. Eduardo Chuluyan, M.D., Ph.D., 3ra Cátedra de Farmacología, Piso 9, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, CP 1121, Buenos Aires, Argentina. E-mail: echuluyan@fmed.uba.ar

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 179. pp 247–253, 2009
Originally Published in Press as DOI: 10.1164/rccm.200804-615OC on November 14, 2008
Internet address: www.atsjournals.org

Secretory leukocyte protease inhibitor (SLPI) is a serine protease inhibitor with activity against cathepsin G, trypsin, and chymotrypsin, but primarily against neutrophil elastase (9). It is constitutively produced by several epithelial cell types and it is induced in response to diverse inflammatory stimuli by various types of cells, such as neutrophils and alveolar macrophages (9–11). On the other hand, the expression and secretion of SLPI are down-modulated by adenoviral infection, transforming growth factor-β_1 cytokine production, and during chronic obstructive lung disease (12–14). In addition, cathepsins B, L, and S and cigarette smoke exposure result in the cleavage and inactivation of SLPI (15, 16). Remarkably, the exposure of murine peritoneal macrophages to heat-killed *M. tuberculosis* (HK-*Mtb*) led to an increase in SLPI gene expression and protein secretion (17). This protein may function as an endogenous immunomodulatory, antiiflammatory, and/or antimicrobial substance (9, 18). In fact, the antimicrobial effects of SLPI against fungi, viruses, and several bacteria have been demonstrated (9). Specifically, the role of SLPI has been shown *in vitro* and *in vivo* against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermidis*, and Group A *Streptococcus* (9). Moreover, Nishimura and colleagues described that recombinant mouse SLPI inhibited the growth of bacillus Calmette-Guèrin and *M. tuberculosis* through the disruption of the mycobacterial cell wall structure, even demonstrating that the N-terminal domain of SLPI was required for the disruption (19). In the present work, we investigated the role of human SLPI on mycobacteria. Our results demonstrated that human SLPI would be a novel soluble PRR that might bind and kill virulent mycobacteria through the direct interaction with ManLAM and PIM, two well known mycobacterium PAMPs.

Some of the results of these studies have been previously reported in the form of an abstract (20).

METHODS

Reagents

Recombinant human SLPI (rhSLPI) was cloned and expressed as described previously (21).

Murine Model of *M. bovis* BCG Pulmonary Infection

BALB/c mice were anesthetized and intranasally infected with *M. bovis* BCG (1×10^6 CFU/mouse). Mice were then intranasally instilled with 50 μl of rhSLPI (150 μg/ml per mouse) and killed according to the protocol described in each figure. Bronchoalveolar lavage fluid and lung tissue were processed as described previously (22) and analyzed for bacterial load. *See* the online supplement for additional details on mice and bacterial load detection.

Antibacterial Assays

The antibacterial activity of rhSLPI was determined with mid–log-phase–grown *M. bovis* BCG-Pasteur and *M. tuberculosis* H37Rv (*see* the online supplement for additional detail on bacterial culture). Bacterial suspensions (1 mg/ml) were treated (16 h, 37°C) with rhSLPI, heat-denatured SLPI (SLPI-Ht), pH-denatured SLPI (SLPI-pH), or control buffer (300 mM NaCl, 50 mM NaH_2PO_4, 2.5 mM imidazole). For kinetic studies, samples were taken every 24 hours for colony-forming unit detection and viable bacteria were detected by plating onto 7H11 agar plates after 2–4 weeks.

SLPI Binding to *M. bovis* BCG

M. bovis BCG suspensions were incubated with rhSLPI, rhSLPI-pH, or rhSLPI-Ht (2 h, 37°C) and then incubated with Penta-His Alexa Fluor 647 conjugate (30 min, 4°C) to detect rhSLPI histidine-tagged protein (21). After washing, the cells were fixed (1.5% paraformaldehyde) and binding was detected by flow cytometry (*see* the online supplement for additional detail on flow cytometry).

SLPI Binding to *Mycobacterium* PAMPs

Tripalmitic acid, trehalose dimycolate (TDM; *see* the online supplement for additional detail on lipid extractions), mannan-capped lipoarabinomannan (ManLAM), phosphatidylinositol mannoside (PIM), mycolic acids, and 19-kD lipoprotein were coated in the wells of a 96-well microplate. After blocking the nonspecific binding sites, rhSLPI (50 ng/ml), rabbit polyclonal anti-human SLPI antibody, goat anti-rabbit peroxidase-conjugated antibody, and colorimetric reactant were sequentially added. The reaction was stopped with H_2SO_4 and the A_{450} was measured with a Multiskan ELISA plate reader (Rayto, Buenos Aires, Argentina).

Phagocytosis of BCG and H37Rv Particles

THP-1 macrophages and murine alveolar macrophages (AMs) were fed with rhSLPI-opsonized fluorescein isothiocyanate (FITC)-conjugated BCG and H37Rv, respectively. Cells were washed and fixed. Fluorescent THP-1 macrophages (i.e., cells that had incorporated BCG–FITC) were analyzed by flow cytometry, whereas AMs were stained with Ziehl-Neelsen to analyze the phagocytosis of H37Rv mycobacteria by microscopy (*see* the online supplement for additional detail on phagocytosis assays, macrophage culture, and labeling of mycobacteria).

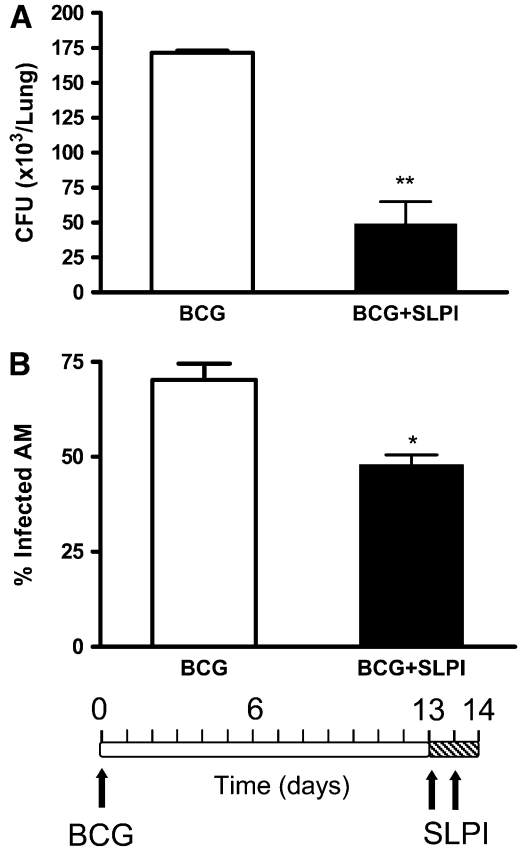

Figure 1. Mycobacterial load from *Mycobacterium bovis* BCG–infected mice treated with recombinant human secretory leukocyte protease inhibitor (rhSLPI). Female BALB/c mice were intranasally infected with 1×10^6 CFU per mouse. Thirteen days postinfection mice were treated twice per day with rhSLPI (150 μg/ml per mouse) or rhSLPI elusion buffer (control) for 2 days. On Day 14, mice were killed and appropriate dilutions of lung homogenates were seeded onto agar plates for colony-forming unit counts and/or stained with Ziehl-Neelsen. In (*A*) colony-forming units per lung and (*B*) percentage of alveolar macrophages (AM) positive for *M. bovis* BCG are shown. The average of 10 fields of vision per lung under oil immersion is shown. *$P < 0.05$, **$P < 0.001$ (n = 6 per group) by Student *t* test.

Detection of SLPI Bound to Mycobacteria in Sputum from Patients with Active Tuberculosis

Sputum samples from 11 patients with tuberculosis (*see* the online supplement for additional detail on patients with active tuberculosis) were heat fixed on glass slides and divided into two sections. After blocking, one section of each glass was incubated with murine IgG1 isotype control and the other with mouse IgG_1 anti-human SLPI monoclonal antibody (2 h, 4°C). After washing and further blocking, the sputum samples were incubated with anti-mouse–FITC polyclonal antibody. Last, the slides were fixed and fluorescent bacilli were photographed as described in the figures.

Statistical Analysis

Student *t* test, analysis of variance, and *post hoc* Bonferroni and Student-Newman-Keuls were used for statistical analysis of the data, as indicated in each figure.

RESULTS

Murine Model of Pulmonary Infection with *M. bovis* BCG-Pasteur

Because SLPI is an endogenous antiinflammatory and antimicrobial substance present in the respiratory tract (10), we investigated the potential role of SLPI during pulmonary mycobacterial infection. Thus, we first analyzed whether SLPI treatment might participate in the outcome of *M. bovis* BCG infection in BALB/c mice. Mice receiving SLPI since Day 13 (twice per day) were killed on Day 14 postinfection and lung bacterial load was determined. (Figure 1A) shows that the number of colony-forming units recovered from BCG-SLPI–treated mice was significantly lower compared with BCG control mice. Furthermore, fewer AM from BCG-SLPI–treated mice had phagocytized mycobacteria compared with control BCG-treated animals (Figure 1B). Together, these results demonstrate that SLPI decreases the *M. bovis* BCG burden once the pulmonary infection has been established.

In Vitro Antimycobacterial Effect of SLPI

We hypothesized that SLPI could act on the pathogen and/or on cells of the immune system of the host. To test this hypothesis, we first analyzed the potential direct effect of SLPI on *Mycobacterium* viability *in vitro.* For this, mid-log–phase–grown *M. bovis* BGC was incubated with various concentrations of SLPI and the bacteria were plated and incubated at 37°C. At various time points *M. bovis* BCG viability was assessed by colony-forming unit counting. Figure 2A shows that SLPI reduced the viability of *M. bovis* BCG in a dose-dependent manner, indicating that SLPI would be in fact acting on the pathogen. Next, we performed time course experiments to determine whether SLPI was also interfering with bacterial growth. Thus, bacterial suspensions (10^5/ml) were incubated with SLPI for 7 days. Samples were taken from BCG cultures every day and bacterial growth was analyzed on agar plates as described previously. As shown in Figure 2B, SLPI gradually and progressively decreased the number of colony-forming units throughout the 7 days studied. Remarkably, the effect of SLPI on mycobacterial viability was also observed with the virulent strain *M. tuberculosis* H37Rv (Figure 2C), further demonstrating that SLPI effectively kills *Mycobacterium* species. The effect of SLPI on mycobacterial viability was not observed when heat-denatured SLPI was used (Figure 2D). However, we still observed that pH-treated SLPI decreased bacterial viability (Figure 2D). Interestingly, both pH- and heat-denatured SLPI lose their ability to inhibit trypsin serine protease activity (data not shown). Thus, these data indicate that the effect of SLPI on mycobacterial viability does not require the COOH-terminal domain, where the serine protease inhibitory activity resides.

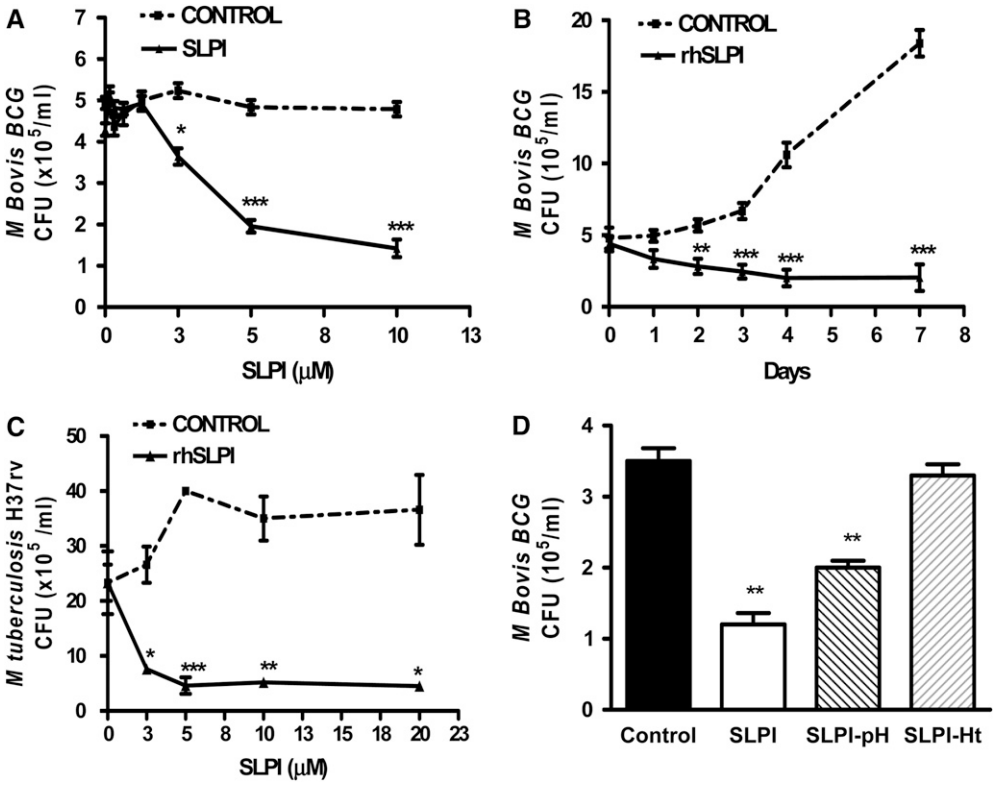

Figure 2. *In vitro* effect of rhSLPI on *M. bovis* BCG and H37Rv viability. (*A*) Fifty-microliter volumes of mid–log-phase bacteria at 1 mg/ml in Sautón buffer were added to 96-well polystyrene microplates in triplicate, together with 50 μl of various concentrations of rhSLPI or control buffer. After overnight incubation at 37°C, the number of colony-forming units was determined by plating serial dilutions onto 7H11 agar plates. For the kinetics study (*B*), 1-ml aliquots of bacterial suspension were incubated with equal volumes of rhSLPI (5 μM) and incubated at 37°C for 7 days. Aliquots (20 μl) were taken every 24 hours to perform colony-forming unit counts. After 2 weeks of incubation, colony-forming unit counts were assessed by visualization under a magnifying glass. The same protocol described in (*A*) was used for experiments with virulent strain *M. tuberculosis* H37Rv (*C*). To examined the effect of pH- and heat-denatured rhSLPI (SLPI-pH and SLPI-Ht, respectively), the peptides were incubated with bacteria for 7 days at 5 μM (*D*). Data represent the mean ± SEM of three separate experiments. $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ compared with control groups, using a Student *t* test (*A*–*C*) and analysis of variance (ANOVA) with Bonferroni test (*D*).

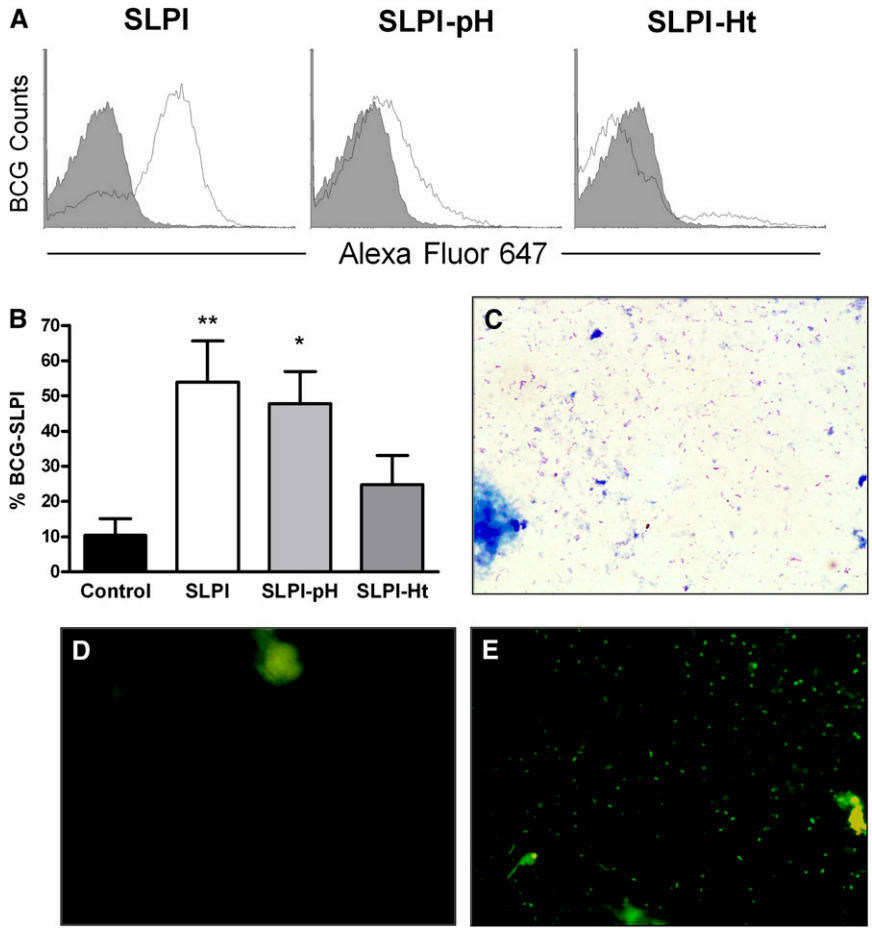

Figure 3. Binding of rhSLPI to *M. bovis* BCG surface. Bacterial particles (1×10^5) were incubated with control buffer, rhSLPI (5 μM), rhSLP-pH (5 μM), or rhSLPI-Ht (5 μM) for 2 hours at 37°C. After incubation, the cells were washed and incubated with Penta-His Alexa Fluor 647 conjugate (30 min, 4°C) to detect histidine-tagged rhSLPI. The cells were fixed and binding was detected by flow cytometry as described in METHODS. A representative experiment is shown in (*A*). Data of five independent experiments are shown in (*B*). The presence of acid- and alcohol-resistant bacilli was determined with Ziehl-Neelsen stain in sputum samples of patients with tuberculosis (*C*). Furthermore, sputum samples were incubated with a control isotype antibody (*D*) or with a monoclonal antibody against human SLPI (E). Fluorescent bacilli were observed and photographed with an Axiophot microscope. The photos show a representative section of a sample (n = 4) under a ×100 oil immersion objective. *$P < 0.05$, **$P < 0.01$ (post-hoc Student Newman Keuls).

Direct Interaction between SLPI and *M. bovis* BCG Cell Envelope

Cationic peptides, like SLPI, kill bacteria by altering the stability of the cell envelope. Thus, we next investigated by flow cytometry the ability of SLPI to attach to the BCG surface. As shown in Figures 3A and 3B, SLPI and SLPI-pH bound to the surface of BCG particles, as demonstrated by a marked increase in mean fluorescence. However, SLPI-Ht was not able to bind to the surface of the bacteria (Figures 3A and 3B). Remarkably, SLPI binding to the *M. tuberculosis* surface was also observed in sputum samples from patients with active tuberculosis (Figures 3D and 3E). To confirm the staining of bacteria, the same slides were analyzed for acid- and alcohol-resistant bacilli by Ziehl-Neelsen (ZN) staining. As shown in Figure 3C, the SLPI fluorescence pattern was quite similar to that of ZN staining. Together, these results clearly demonstrate that SLPI binds directly to bacteria.

Mycobacteria display some well-defined PAMPs required by the host immune system to detect the presence of the bacillus and to trigger an effective immune response (23). Therefore, we next investigated whether SLPI might bind to any of the already known mycobacterial PAMPs. Interestingly, our results showed that SLPI displayed a high binding capacity for TDM and ManLAM, and a lower binding capacity for PIM (Figure 4). In contrast, SLPI did not bind 19-kD lipoprotein or mycolic acids (Figure 4). Together, these results further confirm the capacity of SLPI to specifically bind to known PAMPs from virulent mycobacteria, strongly suggesting that SLPI could act as a PRR during the host immune response against *Mycobacterium* infection.

SLPI Facilitates Phagocytosis of Mycobacterial Strains

To confirm that SLPI is a PRR, we next analyzed the capacity of SLPI to facilitate THP-1 macrophage and murine AM phagocytosis of BCG particles and H37Rv, respectively. BCG particles were first stained with FITC and then coated with rhSLPI.

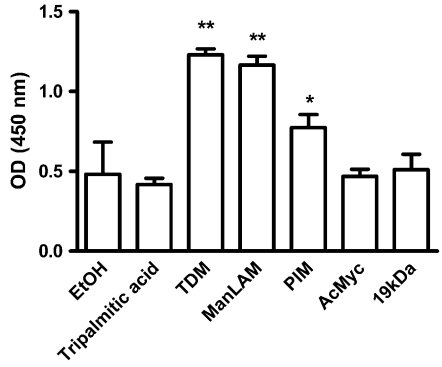

Figure 4. Binding to mycobacterial lipids. Tripalmitic acid, trehalose dimycolate (TDM), mannan-capped lipoarabinomannans (ManLAM), phosphatidylinositol mannoside (PIM), mycolic acids (AcMyc), and 19-kD lipoprotein (19kDa) were dissolved in ethanol (EtOH) and deposited in a 96-well Polysorp microplate as described in METHODS. rhSLPI (5 μM) was then added to the wells and peptide binding was revealed as described in METHODS. Absorbance was read at 450 nm with a Multiskan ELISA plate reader. Data represent the means ± SEM of five independent experiments. *$P < 0.01$, **$P < 0.001$ by ANOVA with Bonferroni test.

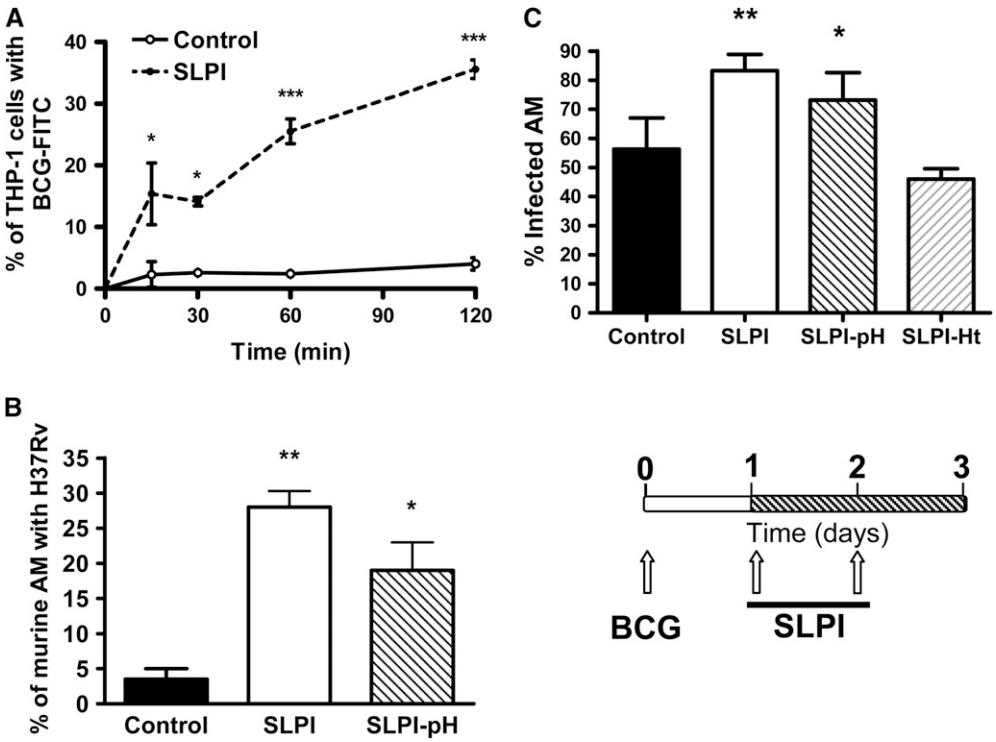

Figure 5. SLPI facilitates phagocytosis of mycobacterial strains. (*A*) THP-1 cells (2×10^5) were incubated with fluorescein isothiocyanate (FITC)-labeled BCG particles (multiplicity of infection, 1:12) opsonized or not with rhSLPI. Fluorescent cells that had incorporated bacteria were analyzed by flow cytometry. Results are expressed as the percentage phagocytosis of BCG particles. (*B*) Murine AMs (1×10^5) were incubated with H37Rv particles (multiplicity of infection, 1:6) opsonized or not with rhSLPI or rhSLPI-pH. Cells were then fixed and stained with Ziehl-Neelsen. Phagocytosis was assessed by light microscopy. Triplicate experiments were performed and at least 100 cells per slide were counted. The percentage of phagocytic cells having ingested at least one bacterium was arbitrarily calculated as follows: number of phagocytic cells/number of total cells $\times$ 100. Values represent the mean $\pm$ SEM of three separate experiments performed in triplicate for (*A*) and (*B*). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (post-hoc Student Newman Keuls). (*C*) Female BALB/c mice infected as in Figure 1 were treated with rhSLPI or control buffer as indicated. On Day 3, mice were killed and lung homogenates were processed as in Figure 1. Data represent the means $\pm$ SEM of three independent experiments. $^*P < 0.05$, $^{**}P < 0.001$ (n = 6 per group; *post hoc* Student Newman Keuls).

In this way, rhSLPI-coated BCG particles were fed to macrophages and uptake was evaluated by flow cytometry. As shown in Figure 5A, over a time period of 120 minutes, phagocytosis of SLPI-coated *M. bovis* BCG was faster than that of uncoated mycobacteria. Figure 5B also shows that phagocytosis of H37Rv by murine AMs was higher when the virulent strain was coated with SLPI or SLPI-pH compared with uncoated H37Rv. These data demonstrated that SLPI is not only able to kill mycobacteria, but that it also can facilitate clearing of the pathogen by the immune system. The role of SLPI as a PRR was also tested and confirmed with an *in vivo* model of murine BCG infection. For this, we performed experiments similar to those described in Figure 1, with modifications. Briefly, SLPI was administered (twice per day) to animals 24 hours after *M. bovis* BCG mouse infection. On Day 3, mice were killed and the number of infected AMs was determined. In contrast to our findings shown in Figure 1, Figure 5C shows that significantly higher numbers of AMs containing BCG were detected in BCG-SLPI– and BCG-SLPI-pH–treated mice compared with control animals. This effect was not observed in BCG-SLPI-Ht–treated mice (Figure 5C). Thus, SLPI increased not only *in vitro* but also *in vivo* bacterial phagocytosis, and the effect does not require the activity of serine protease inhibitory domain.

DISCUSSION

Our results demonstrated that human SLPI displays antimycobacterial activity, therefore confirming that SLPI is an important endogenous agent as such. Moreover, we showed that SLPI is able to attach to and interact with the surface of mycobacteria, increasing the phagocytosis of the pathogen. Furthermore, the present data sustain our hypothesis that SLPI is a novel secreted PRR that might participate in the early phases of a pulmonary infection with virulent mycobacteria.

Many species contain antimicrobial cationic peptides that have a broad spectrum of activity not only against gram-negative and gram-positive bacteria but also against fungi, viruses, and parasites (24). SLPI is a polycationic nonglycosylated peptide that displays antimicrobial properties *in vivo* and *in vitro* (25). The antimicrobial activity of human SLPI has been described for various bacteria as well as *Aspergillus fumigatus*, *Candida albicans*, and HIV (25). The antimycobacterial activity of SLPI resides in the whey-acidic-protein domains of the molecule and is similar to that of other cationic peptides, such as defensins (26). In an attempt to localize the antimycobacterial activity of SLPI, we performed studies with denatured SLPI. In our experiments, SLPI denatured either by heat or pH renders a protease that loses antiprotease activity (data not shown). However, this antiprotease activity is not directly related to the antimycobacterial activity and binding capacity of the protein because pH-treated rhSLPI, but not heat-inactivated rhSLPI (Figure 3B), is able to bind to the mycobacterial surface and maintain the antimycobacterial activity (Figure 2D). It is worth noting that heat inactivation disrupts hydrogen bonds and nonpolar hydrophobic interactions, whereas acids disrupt salt bridges. Within a physiological context, it is of relevance that even after acid pH denaturation SLPI retained its activity against bacteria, indicating that the effect of SLPI on mycobacteria might persist even at inflammatory sites, where the extracellular pH is often decreased (27).

Although we showed the binding of SLPI to the surface of mycobacteria *in vitro*, an important aspect of our study was the ability to demonstrate the attachment of SLPI to *M. tuberculosis* observed in sputum samples of patients with active disease. These results confirm that endogenous SLPI is able to bind to the surface of virulent strains of *Mycobacterium*, although we cannot confirm the origin of the SLPI present in sputum from

patients with tuberculosis, in particular considering that SLPI is found in high concentration in saliva (28).

In the lung, SLPI is an alarm-mediating acute-phase reactant secreted in response to LPS, IL-1, tumor necrosis factor-α, epidermal growth factor, defensins, and human neutrophil elastase (9). The SLPI concentration found in respiratory epithelial lining fluid is about 1 μM (29), a value that is close to the concentration we used to detect the antimicrobial activity of rhSLPI (2.5 μM protein). However, this concentration is higher than the concentration that was used to demonstrate the antimycobacterial activity of mouse SLPI (19). It is probable that the discrepancy in effects of human and mouse SLPI are related to structural differences between the peptides (19). In the present study, we showed that low concentrations of rhSLPI were enough to bind, kill, and induce phagocytosis against mycobacteria. Thus, although the concentration required for the antimicrobial activity of rhSLPI is higher than in mice, it is likely that the binding of SLPI to mycobacteria does not require a high protein concentration.

Pattern recognition receptors evolved to recognize PAMPs on pathogens, and therefore to detect the presence of infection. Several PAMPs have been recognized on *M. tuberculosis* (30). Binding of SLPI to *Mycobacterium* seems to be specific and not related to the electric charge because we observed specific binding to TDM, ManLAM, and PIM but not to mycolic acids and 19-kD lipoprotein. The 19-kD lipoprotein has been identified as a major PAMP, considering its activity to inhibit MHC-II antigen processing. Then, our results clearly indicate that SLPI is a secreted PRR that binds to other major PAMPs on the surface of *Mycobacterium* and that this binding facilitates the clearing by macrophages and subsequent killing of the pathogen. The clearance of bacteria by macrophages is also supported by treating mice with SLPI shortly after BCG challenge (Figure 5C). In fact, this protocol allowed us to detect higher numbers of AM containing BCG compared with AM recovered from control mice. On the other hand, when SLPI was given once infection had been established (Figure 1B), and probably most of the mycobacteria were inside the macrophages, a lower number of AM containing mycobacteria was observed. Besides, the ability of SLPI to function as a PRR for mycobacteria is lost when the protein is denatured with heat but not with pH treatment, either for *in vitro* (Figure 3A) or *in vivo* (Figure 5C) experiments. Moreover, similar to SLPI microbicidal activity, SLPI PRR ability does not depend on the COOH-terminal domain, where the serine protease inhibitory activity resides, because SLPI-pH lacks serine protease activity (data not shown).

Herein we have described for the first time that human SLPI is a secreted PRR for mycobacteria that increases both the phagocytosis and killing of the pathogen. Further experiments are required to unravel the role of SLPI in the pathophysiology of tuberculosis, before it may be considered therapeutically useful in the management of the disease.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Dr. Martha Ambroggi for expert assistance with the samples from patients with tuberculosis. The authors also thank Marcela Amoroso and María Luisa Brero (Bioterio Central del INPB-ANLIS Dr. Carlos G. Malbrán) for technical assistance.

References

1. Kaufmann SH, Cole ST, Mizrahi V, Rubin E, Nathan C. *Mycobacterium tuberculosis* and the host response. *J Exp Med* 2005;201: 1693–1697.
2. Subramanyam S, Hanna LE, Venkatesan P, Sankaran K, Narayanan PR, Swaminathan S. HIV alters plasma and *M. tuberculosis*–induced cytokine production in patients with tuberculosis. *J Interferon Cytokine Res* 2004;24:101–106.
3. Hertoghe T, Wajja A, Ntambi L, Okwera A, Aziz MA, Hirsch C, Johnson J, Toossi Z, Mugerwa R, Mugyenyi P, *et al.* T cell activation, apoptosis and cytokine dysregulation in the (co)pathogenesis of HIV and pulmonary tuberculosis (TB). *Clin Exp Immunol* 2000;122:350–357.
4. Ulrichs T, Kaufmann SH. New insights into the function of granulomas in human tuberculosis. *J Pathol* 2006;208:261–269.
5. Medzhitov R, Janeway CA Jr. Decoding the patterns of self and nonself by the innate immune system. *Science* 2002;296:298–300.
6. Andrews T, Sullivan KE. Infections in patients with inherited defects in phagocytic function. *Clin Microbiol Rev* 2003;16:597–621.
7. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;124:783–801.
8. Doz E, Rose S, Nigou J, Gilleron M, Puzo G, Erard F, Ryffel B, Quesniaux VF. Acylation determines the Toll-like receptor (TLR)-dependent positive versus TLR2-, mannose receptor-, and SIGNR1-independent negative regulation of pro-inflammatory cytokines by mycobacterial lipomannan. *J Biol Chem* 2007;282:26014–26025.
9. Williams SE, Brown TI, Roghanian A, Sallenave JM. SLPI and elafin: one glove, many fingers. *Clin Sci (Lond)* 2006;110:21–35.
10. Saitoh H, Masuda T, Shimura S, Fushimi T, Shirato K. Secretion and gene expression of secretory leukocyte protease inhibitor by human airway submucosal glands. *Am J Physiol Lung Cell Mol Physiol* 2001; 280:L79–L87.
11. van Wetering S, van der Linden AC, van Sterkenburg MA, Rabe KF, Schalkwijk J, Hiemstra PS. Regulation of secretory leukocyte proteinase inhibitor (SLPI) production by human bronchial epithelial cells: increase of cell-associated SLPI by neutrophil elastase. *J Investig Med* 2000;48:359–366.
12. Luo BL, Niu RC, Feng JT, Hu CP, Xie XY, Ma LJ. Downregulation of secretory leukocyte proteinase inhibitor in chronic obstructive lung disease: the role of TGF-β/Smads signaling pathways. *Arch Med Res* 2008;39:388–396.
13. Higashimoto Y, Yamagata Y, Iwata T, Ishiguchi T, Okada M, Masuda M, Satoh H, Itoh H. Adenoviral E1A suppresses secretory leukoprotease inhibitor and elafin secretion in human alveolar epithelial cells and bronchial epithelial cells. *Respiration* 2005;72:629–635.
14. Jaumann F, Elssner A, Mazur G, Dobmann S, Vogelmeier C; Munich Lung Transplant Group. Transforming growth factor-β1 is a potent inhibitor of secretory leukoprotease inhibitor expression in a bronchial epithelial cell line. *Eur Respir J* 2000;15:1052–1057.
15. Taggart CC, Lowe GJ, Greene CM, Mulgrew AT, O'Neill SJ, Levine RL, McElvaney NG. Cathepsin B, L, and S cleave and inactivate secretory leucoprotease inhibitor. *J Biol Chem* 2001;276:33345–33352.
16. Cavarra E, Lucattelli M, Gambelli F, Bartalesi B, Fineschi S, Szarka A, Giannerini F, Martorana PA, Lungarella G. Human SLPI inactivation after cigarette smoke exposure in a new *in vivo* model of pulmonary oxidative stress. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L412–L417.
17. Ding A, Yu H, Yang J, Shi S, Ehrt S. Induction of macrophage-derived SLPI by *Mycobacterium tuberculosis* depends on TLR2 but not MyD88. *Immunology* 2005;116:381–389.
18. Fitch PM, Roghanian A, Howie SE, Sallenave JM. Human neutrophil elastase inhibitors in innate and adaptive immunity. *Biochem Soc Trans* 2006;34:279–282.
19. Nishimura J, Saiga H, Sato S, Okuyama M, Kayama H, Kuwata H, Matsumoto S, Nishida T, Sawa Y, Akira S, *et al.* Potent antimycobacterial activity of mouse secretory leukocyte protease inhibitor. *J Immunol* 2008;180:4032–4039.
20. Gómez S, Arguelles C, Guerrieri D, Slimorich R, Tateosian N, Pasquinelli V, Chuluyan HE, Garcia VE. *In vivo* and *in vitro* bactericidal effects of secretory leukocyte protease inhibitor (SLPI) on *Mycobacterium bovis* bacillus Calmette-Guerin (BCG). Abstract presented at the 13th International Congress of Immunology, August 21–25, 2007, Río de Janeiro, Brazil.
21. Maffia PC, Zittermann SE, Scimone ML, Tateosian N, Amiano N, Guerrieri D, Lutzky V, Rosso D, Romeo HE, Garcia VE, *et al.* Neutrophil elastase converts human immature dendritic cells into transforming growth factor-β1–secreting cells and reduces allostimulatory ability. *Am J Pathol* 2007;171:928–937.
22. Fulton SA, Martin TD, Redline RW, Henry Boom W. Pulmonary immune responses during primary *Mycobacterium bovis* Calmette-Guerin bacillus infection in C57Bl/6 mice. *Am J Respir Cell Mol Biol* 2000;22:333–343.

23. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, Akira S. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol* 2002;169:10–14.
24. Hancock RE, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol* 2006;24:1551–1557.
25. Sallenave JM. Antimicrobial activity of antiproteinases. *Biochem Soc Trans* 2002;30:111–115.
26. Verma C, Seebah S, Low SM, Zhou L, Liu SP, Li J, Beuerman RW. Defensins: antimicrobial peptides for therapeutic development. *Biotechnol J* 2007;2:1353–1359.
27. Simmen HP, Battaglia H, Giovanoli P, Blaser J. Analysis of pH, pO_2 and pCO_2 in drainage fluid allows for rapid detection of infectious complications during the follow-up period after abdominal surgery. *Infection* 1994;22:386–389.
28. Baqui AA, Meiller TF, Falkler WA Jr. Enhanced secretory leukocyte protease inhibitor in human immunodeficiency virus type 1–infected patients. *Clin Diagn Lab Immunol* 1999;6:808–811.
29. Hirsch J, Elssner A, Mazur G, Maier KL, Bittmann I, Behr J, Schwaiblmair M, Reichenspurner H, Fürst H, Briegel J, *et al.* Bronchiolitis obliterans syndrome after (heart-)lung transplantation: impaired antiprotease defense and increased oxidant activity. *Am J Respir Crit Care Med* 1999;160:1640–1646.
30. Tobian AA, Potter NS, Ramachandra L, Pai RK, Convery M, Boom WH, Harding CV. Alternate class I MHC antigen processing is inhibited by Toll-like receptor signaling pathogen–associated molecular patterns: *Mycobacterium tuberculosis* 19-kDa lipoprotein, CpG DNA, and lipopolysaccharide. *J Immunol* 2003;171:1413–1422.