The Journal of Immunology

Potent Antimycobacterial Activity of Mouse Secretory Leukocyte Protease Inhibitor

Junichi Nishimura, Hiroyuki Saiga, Shintaro Sato, Megumi Okuyama, Hisako Kayama, Hirotaka Kuwata, Sohkichi Matsumoto, Toshirou Nishida, Yoshiki Sawa, Shizuo Akira, Yasunobu Yoshikai, Masahiro Yamamoto and Kiyoshi Takeda

J Immunol 2008; 180:4032-4039; ;
doi: 10.4049/jimmunol.180.6.4032
http://www.jimmunol.org/content/180/6/4032

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Potent Antimycobacterial Activity of Mouse Secretory Leukocyte Protease Inhibitor[1]

Junichi Nishimura,*§ Hiroyuki Saiga,*‡ Shintaro Sato,¶ Megumi Okuyama,‖ Hisako Kayama,*‡ Hirotaka Kuwata,* Sohkichi Matsumoto,‖ Toshirou Nishida,§ Yoshiki Sawa,§ Shizuo Akira,¶ Yasunobu Yoshikai,† Masahiro Yamamoto,‡ and Kiyoshi Takeda[2]*‡

Secretory leukocyte protease inhibitor (SLPI) has multiple functions, including inhibition of protease activity, microbial growth, and inflammatory responses. In this study, we demonstrate that mouse SLPI is critically involved in innate host defense against pulmonary mycobacterial infection. During the early phase of respiratory infection with *Mycobacterium bovis* bacillus Calmette-Guérin, SLPI was produced by bronchial and alveolar epithelial cells, as well as alveolar macrophages, and secreted into the alveolar space. Recombinant mouse SLPI effectively inhibited in vitro growth of bacillus Calmette-Guérin and *Mycobacterium tuberculosis* through disruption of the mycobacterial cell wall structure. Each of the two whey acidic protein domains in SLPI was sufficient for inhibiting mycobacterial growth. Cationic residues within the whey acidic protein domains of SLPI were essential for disruption of mycobacterial cell walls. Mice lacking SLPI were highly susceptible to pulmonary infection with *M. tuberculosis*. Thus, mouse SLPI is an essential component of innate host defense against mycobacteria at the respiratory mucosal surface. ***The Journal of Immunology,*** **2008, 180: 4032–4039.**

Mycobacterium tuberculosis is a top killer among bacterial pathogens and is responsible for 2 million deaths annually. The emergence of AIDS and development of multidrug-resistant *M. tuberculosis* have increased the incidence of tuberculosis, and it has now become a serious problem. Therefore, the host defense mechanisms against *M. tuberculosis* have been intensively investigated and important roles of T cell-mediated adaptive immunity are now well established (1, 2). In addition, functional characterization of TLRs has recently indicated the importance of innate immunity in infection with *M. tuberculosis* (3, 4). Macrophages and dendritic cells are the major effectors of TLR-mediated antimycobacterial immune responses, because they produce a variety of proinflammatory cytokines and have the capacity of phagocytosis. However, during *M. tuberculosis* infection, epithelial cells in the respiratory tract as well as alveolar macrophages are the first targets for invasion by *M. tuberculosis*. Therefore, these epithelial cells are expected to play roles in preventing mycobacterial infection by establishing physical barriers and producing proinflammatory and antimicrobial mediators (5).

Secretory leukocyte protease inhibitor (SLPI)[3] is a 12-kDa secreted protein composed of two cysteine-rich whey acidic protein (WAP) domains (also called WAP four-disulfide core (WFDC) domains) (6–8). It was originally identified in seminal fluid and is produced by secretory cells in the genital, respiratory, and lacrimal glands as well as dermal keratinocytes (9–13). SLPI is a potent inhibitor of serine proteases, such as neutrophil elastase and cathepsin G, and has therefore been proposed to protect tissues from protease-mediated damage at sites of inflammation (14, 15). Indeed, SLPI was subsequently shown to mediate wound healing (16, 17). Further studies have revealed that SLPI has additional functions. For example, it possesses an antimicrobial activities against Gram-negative and Gram-positive bacteria, fungi, and viruses, including HIV (18–20). In addition to SLPI, several other serine protease inhibitors containing a single WAP domain, such as Eppin, Elafin, SWAM1, and SWAM2, also possess antimicrobial activities against Gram-negative and Gram-positive bacteria (8, 21, 22). Thus, serine protease inhibitors possessing WAP domains exhibit antimicrobial activities. However, the precise mechanisms by which these serine protease inhibitors exert their antimicrobial activities remain elusive. More recently, SLPI was found to mediate anti-inflammatory responses. Briefly, SLPI is induced in monocytes and macrophages in response to inflammatory stimuli mediated by TLRs (23) and subsequently suppresses TLR-dependent production of inflammatory mediators in macrophages by modulating NF-κB activity (23–25). Consistent with these findings, SLPI-deficient mice are highly sensitive to TLR4 ligand (LPS)-induced endotoxin shock with increased production of IL-6 (26). Thus, SLPI has diverse functions and its precise roles need to be investigated more carefully.

*Deparment of Molecular Genetics and †Division of Host Defense, Research Center for Prevention of Infectious Diseases, Medical Institute of Bioregulation, Kyushu University, Fukuoka; ‡Laboratory of Immune Regulation, Department of Microbiology and Immunology, §Department of Surgery, Graduate School of Medicine, and ¶Department of Host Defense, Research Institute for Microbial Diseases, Osaka University; and ‖Department of Host Defense, Osaka City University Graduate School of Medicine, Osaka, Japan

Received for publication April 3, 2007. Accepted for publication January 9, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare, as well as the Tokyo Biochemical Research Foundation, the Cell Science Research Foundation, the Yakult Bio-Science Foundation, the Osaka Foundation for Promotion of Clinical Immunology, the Sumitomo Foundation, and the Sankyo Foundation of Life Science.

[2] Address correspondence and reprint requests to Dr. Kiyoshi Takeda, Laboratory of Immune Regulation, Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, Suita, Osaka, 565-0871, Japan. E-mail address: ktakeda@ongene.med.osaka-u.ac.jp

[3] Abbreviations used in this paper: SLPI, secretory leukocyte protease inhibitor; WAP, whey acidic protein; WFDC, WAP four-disulfide core; qPCR, quantitative PCR; BALF, bronchoalveolar lavage fluid; BCG, bacillus Calmette-Guérin; FLUOS, 5-(6-)carboxyfluorescein-*N*-hydroxysuccinimide ester; NPN, 1-*N*-phenylnapthylamine; AEC, alveolar epithelial cell.

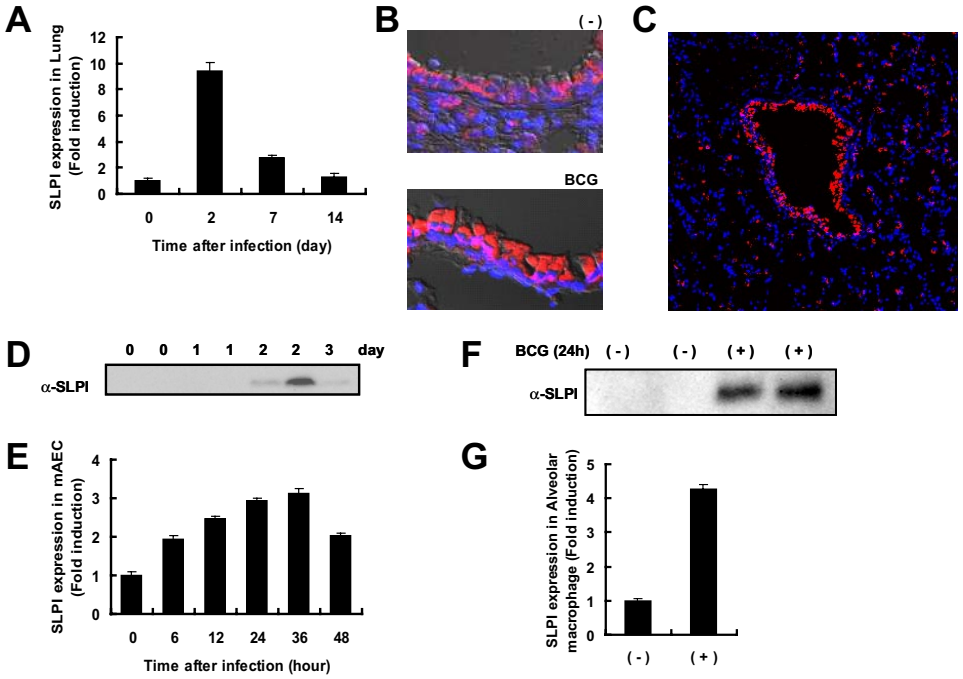

FIGURE 1. Expression of SLPI during mycobacterium infection. *A*, Wild-type mice were intratracheally infected with BCG (4×10^5 CFU). At the indicated periods, total RNA was extracted from the lungs. SLPI mRNA expression was analyzed by quantitative real-time RT-PCR. Data are shown as the relative mRNA levels normalized by the corresponding 18S rRNA level. *B* and *C*, At 2 days after intratracheal infection with BCG, lung tissue sections were stained with an anti-SLPI Ab (red) and 4′,6-diamidino-2-phenylindole (blue) and visualized by fluorescence microscopy. *D*, BALF was collected at the indicated periods after BCG infection. Mouse SLPI protein expression was analyzed by Western blotting with an anti-SLPI Ab. Data obtained from two independent mice (0, 1, and 2 days) are indicated. *E*, AEC were incubated with the same number of BCG for the indicated periods. SLPI mRNA expression was analyzed by quantitative real-time RT-PCR. Data are shown as the relative mRNA levels normalized by the corresponding 18S rRNA level. *F*, AEC were incubated with the same number of BCG. Culture supernatants were collected before (−) and after 24 h of infection (+) and subjected to Western blot analysis using an anti-SLPI Ab. Data obtained from two independent cell clones are shown. *G*, Alveolar macrophages were collected from uninfected wild-type mice, cultured with or without BCG for 48 h, and then analyzed for their SLPI mRNA expression by quantitative real-time RT-PCR. The results are presented as the mean ± SD.

In this study, we investigated the roles of murine SLPI in the context of host defenses against mycobacteria, since SLPI expression is greatly induced in macrophages and the lungs during mycobacterial infection (27). Recombinant SLPI inhibited mycobacterial growth at a lower concentration than that required to inhibit bacterial growth. Inhibition of mycobacterial growth was mediated by increased permeability of the mycobacterial membrane. Mutation of cationic residues in the WAP domains of SLPI resulted in loss of its antimycobacterial activity. Furthermore, SLPI-deficient mice were highly susceptible to pulmonary infection with *M. tuberculosis*. These findings demonstrate that SLPI is a potent antimycobacterial molecule.

Materials and Methods

Cells and bacteria

M. tuberculosis strains H37Ra (ATCC 25177; American Type Culture Collection) and *M. tuberculosis* strains H37Rv (28) were grown in Middlebrook 7H9-ADC medium for 2 wk and stored at −80°C until use. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG; Tokyo strain) was purchased from Kyowa Pharmaceuticals. *Salmonella enterica s*erovar *typhimurium* were provided by the Research Institute for Microbial Diseases (Osaka University). For each experiment, the dose was confirmed by plating an aliquot of the injected bacterial suspension. Isolation and immortalization of type II alveolar epithelial cells from the lungs of transgenic H-2K^b-tsA58 mice were performed as previously described (29), with some modifications.

Immunohistochemistry

Lungs were washed with PBS and frozen in Tissue-Tex OCT compound (Sakura, Tokyo, Japan). Cryostat sections (5-μm thick) were fixed with cold acetone for 10 min, dried, rehydrated with PBS, and blocked with PBS containing 20 mM HEPES, 10% FBS, and 1 μg of Fc-blocking mAb (2.4G2; BD Pharmingen). Next, the sections were sequentially incubated with a biotinylated anti-mouse SLPI Ab (R&D Systems) and Alexa Fluor 594-conjugated streptavidin (Molecular Probes). The nuclei were stained with 4′,6-diamidino-2-phenylindole (Molecular Probes). After washing with PBS, the sections were analyzed by confocal microscopy (Zeiss).

Western blot analysis

Samples were boiled for 5 min in reducing SDS-PAGE sample buffer and then subjected to SDS-PAGE. The separated proteins were transferred to a 0.45-μm pore polyvinylidene fluoride membrane (Millipore). After blocking with 5% milk, the membrane was incubated with the above-described biotinylated anti-mouse SLPI Ab (0.2 μg/ml) and a streptavidin-HRP complex (1/10,000 dilution; R&D Systems). The bound Abs were detected by the Super Signal reagent (Pierce).

Quantitative real-time RT-PCR

After isolation of total RNA with the TRIzol reagent (Invitrogen Life Technologies), 4 μg of the RNA was treated with RQ1DNase (Promega) and then reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) and Random Primers (Toyobo). Gene expression was quantified with an Applied Biosystems PRISM 7000 sequence detection system using TaqMan Universal PCR Master Mix (Applied Biosystems). To determine the relative expression level of each sample, the corresponding 18S rRNA expression level was measured as an internal control. The primer and probe sequences for SLPI were follows: quantitative PCR (qPCR) primer (forward), 5′-d(GCTGTGAGGGTATATGTG GGAAA)-3′; qPCR primer (reverse), 5′-d(CGCCAATGTCAGGGAT CAG)-3′; and qPCR probe, 5′-FAMd(TCTGCCTGCCCCCGATGTG AG)BHQ-3′.

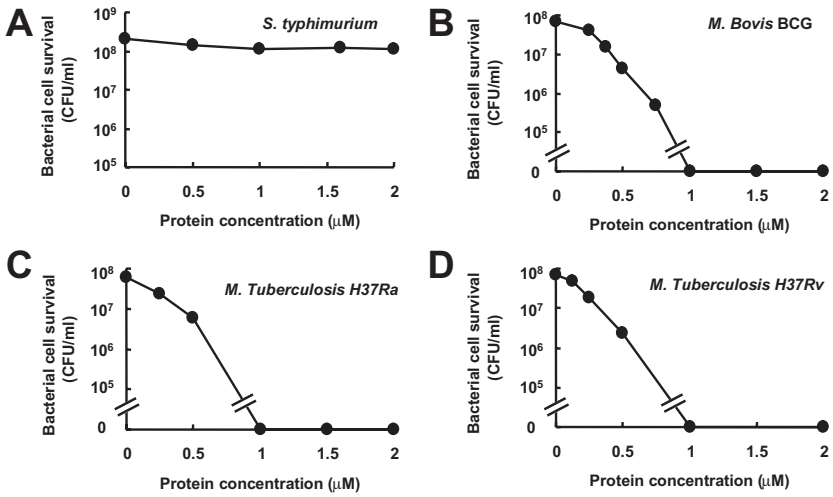

FIGURE 2. Mouse recombinant SLPI inhibits in vitro BCG and *M. tuberculosis* growth. *A*, *S. typhimurium* (5×10^7 CFU/ml) were incubated with SLPI for 2 h and plated on LB agar plates. *B–D*, BCG (*B*), *M. tuberculosis* H37Ra (*C*), or *M. tuberculosis* H37Rv (*D*; 5×10^7 CFU/ml) were incubated with increasing concentrations of recombinant mouse SLPI for 24 h and then plated on 7H10 agar plates.

Bronchoalveolar lavage fluid (BALF)

Mice were intratracheally administered 4×10^5 CFU of BCG suspended in 30 μl of PBS. BALF was collected at the indicated periods. To obtain alveolar macrophages, BALF was centrifuged at 2000 $\times$ g for 2 min and the pellet was resuspended in RPMI 1640 containing 4% FBS. The cell count of alveolar macrophages was $\sim 1 \times 10^5$ cells/mouse. To eliminate contamination by bacteria, alveolar macrophages were cultured with 50 U/ml penicillin and 50 μg/ml streptomycin for 16 h, washed five times, and infected with 5×10^7 CFU/well of BCG without penicillin and streptomycin.

Preparation of recombinant SLPI protein and variants

PCR-amplified mouse SLPI cDNA fragments were inserted into pGEX-6P-1 (Amersham Biosciences). pGEX-6P-1 containing mouse SLPI cDNA was transformed into *Escherichia coli* Rosetta-gami B (DE 3). Expression of GST-SLPI fusion proteins was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactoside, and the expressed fusion proteins were purified using glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions. The purified proteins were incubated with PreScission Protease (Amersham Biosciences) at 4°C for 16 h to cleave the GST tag and then purified with glutathione-Sepharose 4B.

Antibacterial activity

Mid-log phase *Salmonella typhimurium* were diluted with PBS containing 1% Luria-Bertani (LB) to give $\sim 5 \times 10^7$ CFU/ml. A final volume of 250 μl was used to examine the antibacterial activities of proteins. After incubation for 2 h, *S. typhimurium* were plated onto LB agar plates. Colonies were counted (CFU/ml) after overnight incubation at 37°C.

Antimycobacterial activity

M. tuberculosis and BCG were grown in Middlebrook 7H9-ADC medium at 37°C with vigorous agitation. After 7 days of incubation, rapidly growing mycobacteria were harvested by centrifugation and adjusted to 5×10^7 CFU/ml in 7H9-ADC medium. After incubation of the mycobacteria with the indicated concentrations of proteins for 24 h at 37°C, serial 20-fold dilutions were conducted in PBS. Aliquots (50 μl) of the dilutions were plated on Middlebrook 7H10 agar plates and incubated at 37°C for 21–28 days. Colonies were counted (CFU/ml) at intervals until no new colonies appeared.

Protein-binding assay

SLPI and BSA were labeled with 5-(and 6-)carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Roche Diagnostics) as described previously (30). Briefly, 400 μg/ml SLPI or BSA was mixed with 0.096 mg of FLUOS in 1 ml of PBS for 2 h at room temperature. Nonreacted FLUOS was separated by gel filtration using a Sephadex G25 column (Amersham Biosciences). The labeled SLPI or BSA was then incubated with BCG, and the OD at 630 nm was adjusted to 0.2. After 30 min of incubation at 37°C, BCG were washed three times with 7H9 medium containing 0.05% Tween 80. Protein-BCG reactions were detected by confocal laser microscopy (Zeiss).

Scanning electron microscopy

After culture with or without 1 μM SLPI for the indicated times, BCG cultures were fixed with 5% glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated with ethyl alcohol, treated with isoamyl acetate to replace the alcohol, dried with liquid CO_2 in a critical-point apparatus (HCP-2; Hitachi), and coated with Pt-Pd by ion sputtering (Hitachi) in ion-distilled water. The specimens were analyzed using S-4700 scanning electron microscope (Hitachi), operated at 10 kV.

Outer membrane permeabilization assay

The ability of proteins to permeabilize the outer membranes of BCG was investigated using 1-*N*-phenylnaphthylamine (NPN; Wako Pure Chemical Industries) as described previously (31). Briefly, BCG were suspended in 5 mM HEPES (pH 7.4) containing 10 μM NPN to an OD at 590 nm of 0.15. After incubation at 37°C for 30 min, proteins were added and the fluorescence of NPN was monitored. The excitation wavelength used was 340 nm, and the emission wavelength was 425 nm. The experiment was conducted at 37°C.

Generation of $Slpi^{-/-}$ *mice*

The *Slpi* gene was isolated from genomic DNA extracted from embryonic stem cells (E14.1) by PCR using TaKaRa LA *Taq*. The targeting vector was constructed by replacing a 1.2-kb fragment containing exons 2–4 with a neomycin-resistance gene cassette (*neo*) driven by the PGK promoter and inserting a HSV thymidine kinase into the genomic fragment for negative selection. After transfection of the targeting vector into embryonic stem cells, colonies resistant to both G418 and ganciclovir were selected and screened by PCR and Southern blotting. Homologous recombinants were microinjected into blastocysts of C57BL/6 female mice and heterozygous F_1 progenies were intercrossed to obtain $Slpi^{-/-}$ mice.

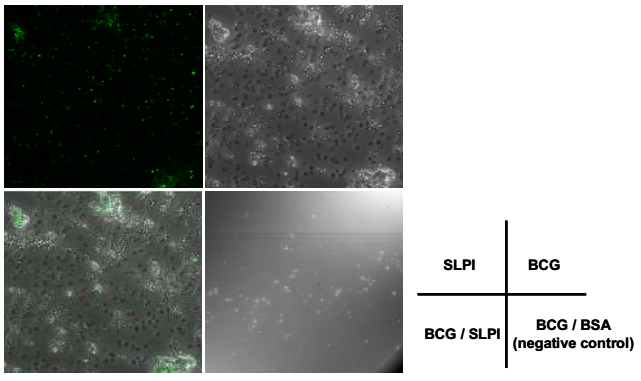

FIGURE 3. SLPI associates with BCG. SLPI and BSA were labeled with FLUOS (Roche). Labeled proteins were incubated with BCG for 30 min, and analyzed by fluorescence microscopy.

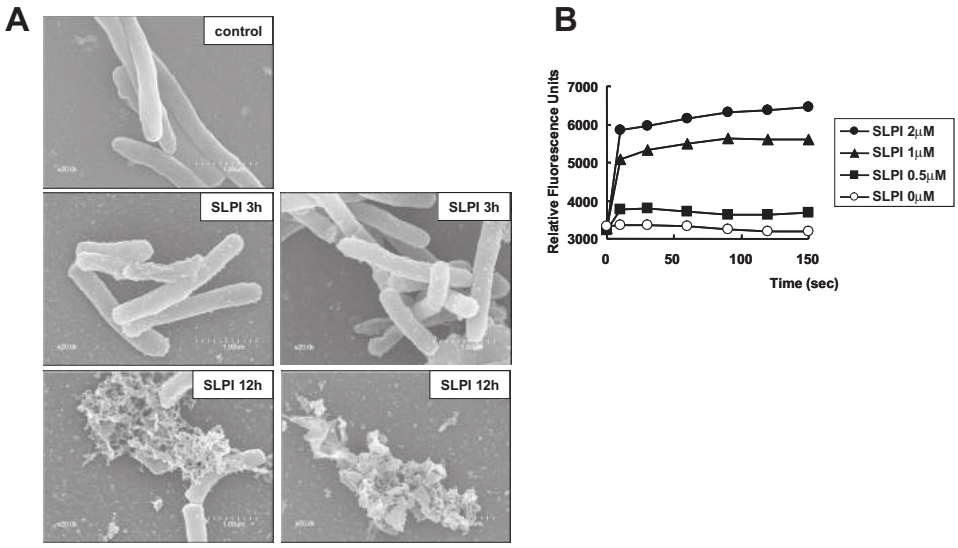

FIGURE 4. SLPI disrupts the BCG cell membrane. *A*, BCG was incubated with or without SLPI for the indicated periods and observed with scanning electron microscopy. *B*, The indicated concentrations of SLPI were added to a BCG suspension containing NPN, and the NPN fluorescence was monitored for the indicated periods. Representative data of three independent experiments are shown.

Slpi$^{-/-}$ mice were backcrossed to C57BL/6 mice for five generations, and *Slpi*$^{-/-}$ and their wild-type littermates from these intercrosses were used for experiments at 6–8 wk of age. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kyushu University.

In vivo infection

For intratracheal infection, 4×10^5 CFU of *M. tuberculosis* suspended in 30 μl of sterile PBS were administered intratracheally. For i.v. infection, 4×10^5 CFU of *M. tuberculosis* suspended in 100 μl of sterile PBS were administered i.v. At 3 wk after infection, homogenates of the lungs and spleen were plated on 7H10 agar plates. For histological examination, 1×10^7 CFU of *M. tuberculosis* suspended in 30 μl of sterile PBS were administered intratracheally. At 5 days after infection, the lungs were fixed in 4% formalin, embedded in paraffin, cut into sections, and stained with H&E.

Results

SLPI expression in the lungs of BCG-infected mice

To assess the roles of SLPI in mycobacterial infection, we first analyzed SLPI expression in the lungs of mice intratracheally infected with *M. bovis* BCG. Total RNA was extracted from the lungs after 2, 7, and 14 days of infection and analyzed for SLPI mRNA expression by real-time qPCR (Fig. 1*A*). Expression of SLPI mRNA was increased by ~9-fold after 2 days of infection, but decreased thereafter. Next, we analyzed pulmonary cell types expressing SLPI by immunohistochemical analysis (Fig. 1, *B* and *C*). SLPI was detected in bronchial epithelial cells before BCG infection (Fig. 1*B, upper micrograph*). After 2 days of BCG infection, increased amounts of SLPI expression were observed, and mainly localized at the apical side of bronchial epithelial cells (Fig. 1*B, lower micrograph*). This prompted us to investigate whether SLPI was secreted into the alveolar space after BCG infection. Accordingly, BALF was collected from BCG-infected mice and analyzed for SLPI protein expression by Western blotting (Fig. 1*D*). SLPI was not detected in BALF from uninfected mice. After 2 days of BCG infection, SLPI was abundantly detected in BALF from infected mice, indicating that SLPI was secreted into the alveolar space during the early phase of mycobacterial infection. In addition to bronchial epithelial cells, SLPI was expressed in cells of the alveolar area (Fig. 1*C*). Therefore, we isolated type II alveolar epithelial cells (AEC) and alveolar macrophages and analyzed their SLPI expression levels after BCG infection. Since AEC are difficult to culture in vitro, we took advantage of transgenic mice harboring a temperature-sensitive mutation of the SV40 large tumor Ag gene under the control of an IFN-γ-inducible H-2K^b promoter element (32, 33). Using these mice, we successfully established AEC lines expressing surfactant protein C (data not shown).

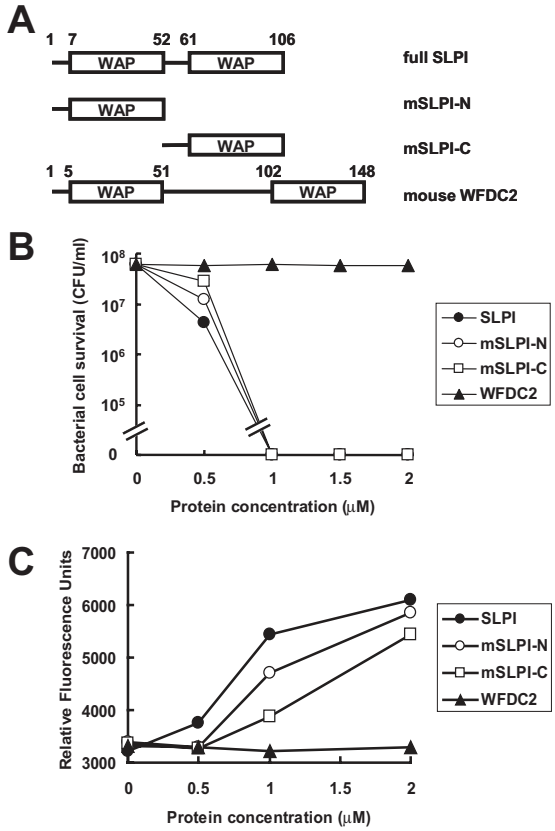

FIGURE 5. A single WAP domain in SLPI is sufficient to inhibit BCG growth. *A*, The deletion mutant constructs mSLPI-N and mSLPI-C lack the C-terminal and N-terminal portions, respectively. White boxes denote WAP domains. *B*, BCG (5×10^7 CFU/ml) was incubated with increasing concentrations of the deletion mutants (mSLPI-N and mSLPI-C) or WFDC2 for 24 h and then plated on 7H10 agar plates. *C*, The indicated concentrations of the deletion mutants (mSLPI-N and mSLPI-C) or WFDC2 were added to BCG suspensions containing NPN. The peak of NPN fluorescence within 150 s was plotted. Representative data of three independent experiments are shown.

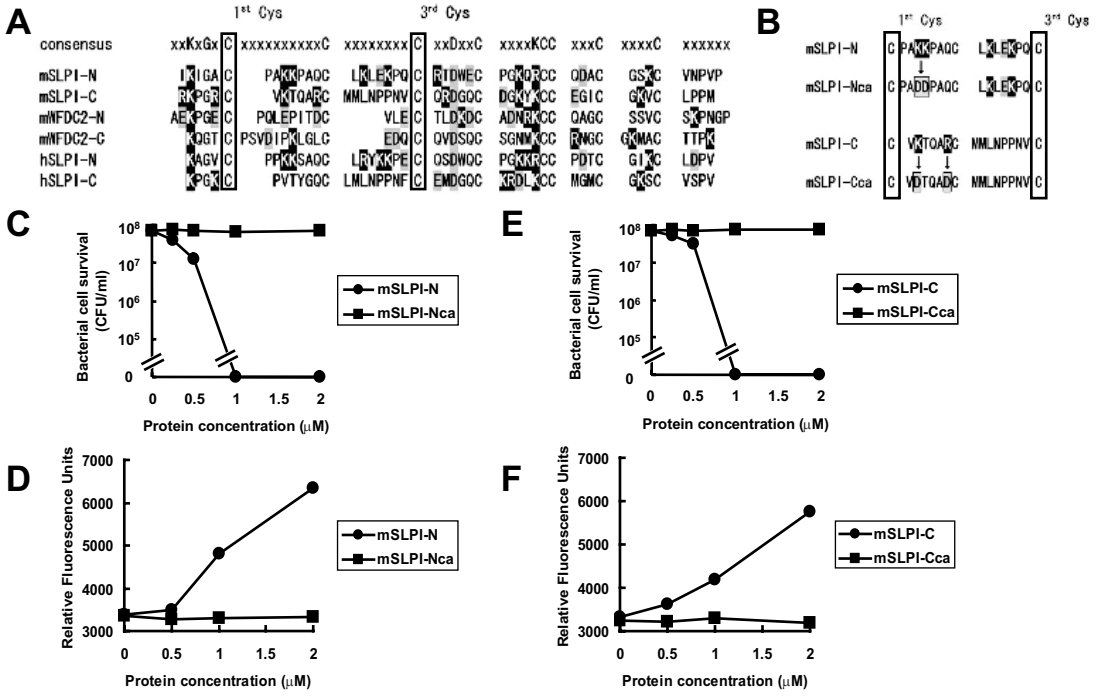

FIGURE 6. Cationic amino acids are responsible for the antimycobacterial activity of SLPI. *A*, Comparison of the WAP domain of SLPI with the WAP domains of other proteins. The consensus amino acid sequence of the WAP domain is shown at the *top* of the protein sequences. Black- and gray-boxed amino acids indicate cationic and anionic amino acids, respectively. Two conserved cysteine residues (first cysteine and third cysteine) are boxed. *B*, Amino acid sequences of the mSLPI-N (mSLPI-Nca) and mSLPI-C (mSLPI-Cca) mutants. *C* and *E*, BCG (5×10^7 CFU/ml) was incubated with increasing concentrations of mSLPI-Nca (*C*) and mSLPI-Cca (*E*) for 24 h and then plated on 7H10 agar plates. *D* and *F*, The indicated concentrations of mSLPI-Nca (*D*) and mSLPI-Cca (*F*) were added to BCG cultures containing NPN. The peak of NPN fluorescence within 150 s was plotted.

AEC were infected with BCG and analyzed for SLPI mRNA expression (Fig. 1*E*). SLPI mRNA expression was gradually induced after BCG infection and peaked after 36 h of infection. AEC have the ability to secrete several effector molecules into the alveolar space. Therefore, we analyzed the SLPI protein levels in culture supernatants from BCG-infected AEC by Western blotting (Fig. 1*F*). SLPI protein was not detected in supernatants from uninfected AEC, but was clearly detected in supernatants after 24 h of BCG infection. Next, isolated alveolar macrophages were infected with BCG and analyzed for SLPI mRNA expression (Fig. 1*G*). BCG infection resulted in an increase in SLPI mRNA expression. Taken together, mycobacterial infection induces the production and secretion of SLPI into the alveolar space by bronchial and type II alveolar epithelial cells as well as alveolar macrophages in the lung.

SLPI-mediated inhibition of mycobacterial growth

Several previous reports have described antimicrobial activities of SLPI against Gram-positive bacteria, Gram-negative bacteria, HIV, and fungi (18–20). However, SLPI needs to be present at high concentrations (>10 μM) for effective inhibition of microbial growth, particularly *S. typhimurium* and *E. coli* (18, 34). Indeed, addition of 2 μM recombinant mouse SLPI only moderately decreased the growth of *S. typhimurium* (Fig. 2*A*). In sharp contrast to the mild inhibition of *S. typhimurium* growth, addition of lower concentrations of mouse SLPI to BCG cultures dramatically reduced the number of CFU (Fig. 2*B*). Growth of BCG was almost completely inhibited by the addition of 1 μM SLPI. A similar inhibitory effect was observed on the growth of *M. tuberculosis* H37Ra and H37Rv (Fig. 2, *C* and *D*). These findings indicate that SLPI has a more potent antimicrobial activity against mycobacteria than against *S. typhimurium*.

Disruption of the BCG cell wall structure by SLPI

Next, we investigated the mechanism of the antimycobacterial activity of SLPI. First, fluorescence-labeled SLPI was incubated with BCG and analyzed by confocal laser microscopy (Fig. 3). BCG and labeled SLPI were colocalized, suggesting that SLPI becomes associated with BCG. We then examined the morphological effects of SLPI on BCG. BCG was incubated with or without SLPI and analyzed by scanning electron microscopy (Fig. 4*A*). BCG exposed to SLPI for 3 h showed pronounced surface blebbing. After 12 h of incubation, many of BCG were collapsed and few live BCG had rough and irregular membrane surfaces. Next, BCG was subjected to an outer membrane permeabilization assay using a fluorescent dye that is weakly fluorescent in aqueous environments but becomes strongly fluorescent in the hydrophobic environment within the cell membrane (Fig. 4*B*). Addition of SLPI caused rapid increases in fluorescence in a dose-dependent manner. These results suggest that SLPI directly associates with mycobacteria, and disrupts the cell wall structure.

Critical role of cationic amino acids in SLPI in its antimycobacterial activity

We next investigated the critical domain involved in the antimycobacterial activity of SLPI. SLPI has two WAP domains (Fig. 5*A*). Several serine protease inhibitors possessing a single WAP domain, such as Eppin, Elafin, SWAM1, and SWAM2, have antimicrobial activities against bacteria such as *E. coli* and *Staphylococcus aureus* (8, 21, 22). To investigate whether each of the WAP domains of mouse SLPI is sufficient to exert antimycobacterial activity, two deletion mutants of SLPI, mSLPI-N and

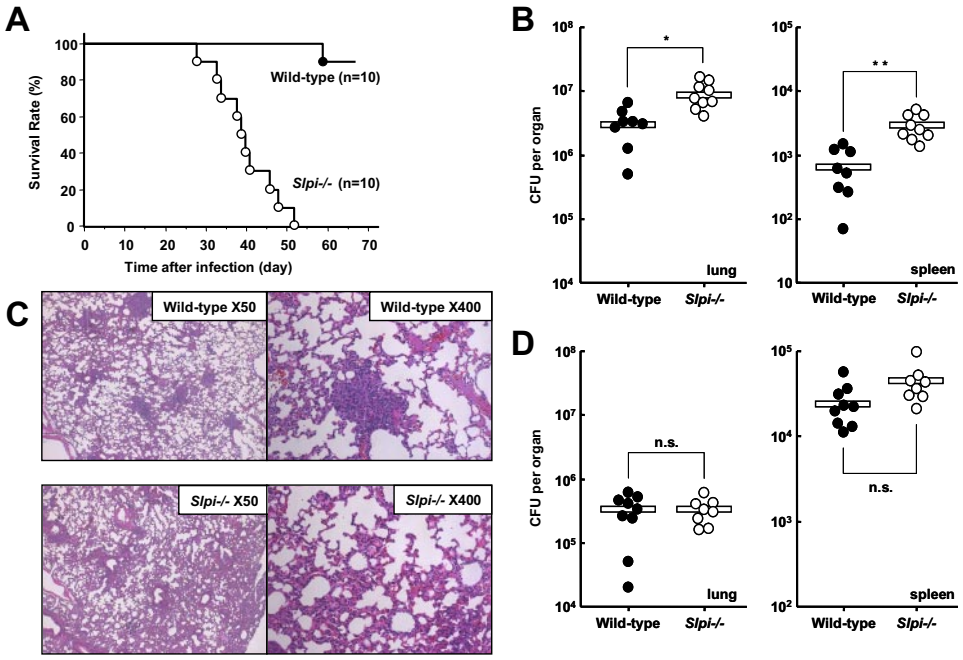

FIGURE 7. *Slpi*$^{-/-}$ mice are highly susceptible to *M. tuberculosis* infection. *A*, *M. tuberculosis* (4×10^5 CFU) were intratracheally infected into wild-type and *Slpi*$^{-/-}$ mice and their survival was monitored. *B*, *M. tuberculosis* (4×10^5 CFU) were intratracheally infected into wild-type and *Slpi*$^{-/-}$ mice. At 3 wk after infection, homogenates of the lungs and spleen were plated on 7H10 agar plates and the CFU titers were counted. Symbols represent individual mice and bars represent the mean of CFU numbers. Statistical analyses were performed using Student's *t* test: *, $p < 0.005$ and **, $p < 0.0005$, significant difference between wild-type and *Slpi*$^{-/-}$ mice. *C*, H&E staining of representative lung tissues from wild-type and *Slpi*$^{-/-}$ mice on day 5 after intratracheal infection with *M. tuberculosis*. *D*, *M. tuberculosis* (4×10^5 CFU) were i.v. infected into wild-type and *Slpi*$^{-/-}$ mice. At 3 wk after infection, homogenates of the lungs and spleen were plated on 7H10 agar plates, and the CFU titers were counted. Symbols represent individual mice and bars represent the mean of CFU numbers. Statistical analyses were performed using Student's *t* test. n.s., Not significant.

mSLPI-C, were generated (Fig. 5*A*). mSLPI-N contained the N-terminal WAP domain, while mSLPI-C contained the C-terminal WAP domain. Both mSLPI-N and mSLPI-C inhibited BCG growth, although their efficiencies were slightly decreased compared with that of full-length SLPI (Fig. 5*B*). Similarly, mSLPI-N and mSLPI-C both induced permeabilization of the outer membrane of BCG with slightly lower efficacies (Fig. 5*C*). These results imply that each WAP domain of mouse SLPI exhibits antimycobacterial activity by disrupting the mycobacterial cell wall structure. WFDC2 is a secreted protein possessing two WAP domains (Fig. 5*A*) (35). However, recombinant mouse WFDC2 had no effect on mycobacterial growth and did not induce permeabilization of the BCG cell membrane, indicating that not all WAP domain-containing proteins have antimicrobial activities (Fig. 5, *B* and *C*). In addition, the N-terminal, but not the C-terminal, WAP domain of human SLPI has been shown to mediate its antimicrobial activities against *E. coli* and *S. aureus* (18). Therefore, we compared the amino acid sequences of the WAP domains of mouse and human SLPI as well as mouse WFDC2 (Fig. 6*A*). The C-terminal regions were conserved among all of the WAP domains. However, the sequences between the first and third cysteine residues were less conserved. In particular, when we examined the sequences between the first and second cysteine residues, we noted that the WAP domains possessing antimycobacterial activities (mSLPI-N, mSLPI-C, and hSLPI-N) contained two or more cationic amino acids, whereas the WAP domains with no antimycobacterial activities (mWFDC2-N, mWFDC2-C, and hSLPI-C) had one or zero cationic acids and instead contained anionic amino acids. Therefore, we produced mSLPI-N (mSLPI-Nca) and mSLPI-C (mSLPI-Cca) mutants, in which the two cationic amino acids were changed to the anionic amino acid aspartic acid (Fig. 6*B*). Neither mSLPI-Nca nor mSLPI-Cca was able to inhibit BCG growth or permeabilize the cell membrane (Fig. 6, *C–F*). These results suggest that the cationic acids of mouse SLPI are responsible for its potent antimycobacterial activities.

High susceptibility of SLPI-deficient mice to M. tuberculosis *infection*

In the next experiment, we assessed the physiological roles of SLPI during mycobacterial infection by generating mice lacking SLPI (*Slpi*$^{-/-}$ mice) via gene targeting (data not shown). First, wild-type and *Slpi*$^{-/-}$ mice were intratracheally infected with *M. tuberculosis* H37Ra, and monitored for their survival (Fig. 7*A*). All *Slpi*$^{-/-}$ mice died within 8 wk of infection at a dose that almost all wild-type mice survived for >9 wk. Next, we counted CFU numbers in the lungs and spleen after 3 wk of infection (Fig. 7*B*). The CFU titers of *M. tuberculosis* in both tissues were higher for *Slpi*$^{-/-}$ mice than that for wild-type mice. The histopathological changes in the lungs after 5 days of *M. tuberculosis* infection were also analyzed (Fig. 7*C*). In wild-type mice, the formation of several small granulomas was observed. In contrast, granulomatous changes were induced to a lesser extent in *Slpi*$^{-/-}$ mice and rather diffuse cell infiltration was observed instead. Next, mice were i.v. infected with *M. tuberculosis*, and the CFU numbers in the lungs and spleen were counted after 3 wk of infection (Fig. 7*D*). The CFU titers were not as dramatically increased in both tissues of *Slpi*$^{-/-}$ mice compared with the corresponding titers in the tissues of wild-type mice, indicating that *Slpi*$^{-/-}$ mice are not highly susceptible to i.v. *M. tuberculosis* infection. Taken together, these findings indicate that *Slpi*$^{-/-}$ mice are highly vulnerable to *M. tuberculosis* infection via the respiratory route.

Discussion

In the present study, we analyzed the roles of mouse SLPI in host defense against mycobacteria. During the early phase of respiratory mycobacterial infection, SLPI was produced and secreted into the alveolar space by bronchial and type II alveolar epithelial cells as well as alveolar macrophages. Recombinant mouse SLPI inhibited the growth of mycobacteria more effectively than it inhibited the growth of Gram-negative bacteria. The SLPI-mediated inhibition of mycobacterial growth was attributable to disruption of the mycobacterial cell wall structure. Furthermore, *Slpi*$^{-/-}$ mice were highly susceptible to pulmonary *M. tuberculosis* infection, highlighting a mandatory role for mouse SLPI in the host defense against *M. tuberculosis* infection. Thus, mouse SLPI is a critical antimycobacterial molecule that acts during the early phase of mycobacterial infection at the respiratory mucosal surface.

Similar structural changes to those observed in SLPI-treated mycobacterial cell walls were induced in several bacteria and *M. tuberculosis* treated with the antimicrobial peptides defensins, which permeabilize microbial membranes (36, 37). We further identified the critical elements for the potent antimycobacterial activity of mouse SLPI. It has been proposed that defensins containing positively charged amino acid residues associate with microorganisms by targeting the surface-exposed negatively charged phospholipid head groups in the microbial membrane (37). Indeed, mutations that change arginine to aspartic acid can attenuate the bactericidal activity of the α-defensin cryptdin-4 (38). Therefore, we supposed that SLPI, which has similar effects on mycobacterial membranes to defensins, also associates with negatively charged mycobacterial membranes through its positively charged amino acid residues. Consistent with this hypothesis, the sequences between the first and second conserved cysteine residues of the WAP domains are not conserved. Moreover, there are several positively charged amino acids (lysine and arginine) in these regions of the WAP domains that possess antimicrobial activities, whereas the regions without any antimicrobial activities contain one or zero positively charged amino acids. Furthermore, structural studies have revealed that the region between the first and second conserved cysteine residues is exposed on the outside of the molecule, thereby enabling this region to associate with microbial membranes (39, 40). Indeed, mutations of the cationic amino acid residues within this region resulted in elimination of the antimycobacterial activity. Thus, mouse SLPI exhibits antimycobacterial activity in quite a similar manner to that of defensins.

In comparison to SLPI, higher concentrations of other serine protease inhibitors containing a WAP domain are required to inhibit microbial growth (8, 21, 22). Recombinant human SLPI is less effective at inhibiting the growth of mycobacteria and *S. typhimurium* (our unpublished data). These differential properties may be attributable to structural differences in the WAP domains, which mediate the antimicrobial activity. SLPI has two WAP domains, whereas other serine protease inhibitors, such as Eppin, Elafin, and SWAMs, have only a single WAP domain. In the case of human SLPI, only the N-terminal WAP domain exhibits antimicrobial activity (18). In addition, only the N-terminal WAP domain of human SLPI contains critical cationic acid residues. The presence of two WAP domains possessing antimicrobial activity may be responsible for the high potency of mouse SLPI for mycobacterial growth inhibition.

Mouse SLPI inhibited mycobacterial growth at profoundly lower concentrations than those required to inhibit the growth of *S. typhimurium* or other microorganisms (18–20). It remains unclear how SLPI becomes more specifically targeted toward mycobacteria. Differential antimicrobial properties against distinct microorganisms have not been reported in the case of defensins. Therefore, SLPI, which has multifunctional properties, may have an unknown strategy for specifically recognizing mycobacteria.

The in vitro findings demonstrating that mouse SLPI inhibits mycobacterial growth were further strengthened by in vivo studies using *Slpi*$^{-/-}$ mice. *Slpi*$^{-/-}$ mice were highly susceptible to pulmonary *M. tuberculosis* infection, but not to i.v. infection. In accordance with this finding, SLPI protein was abundantly detected in the alveolar space after pulmonary BCG infection, but was not detected in sera from mice after i.v. BCG infection (our unpublished data). Therefore, high concentrations of SLPI are supposed to be secreted into the alveolar space during the early phase of respiratory infection with *M. tuberculosis*, thereby promptly killing the mycobacteria before they can invade the lung tissues through the epithelial barrier. Given that mouse SLPI has potent antimycobacterial activities, it would be a good candidate for treatment during the acute phase of *M. tuberculosis* infection and may even be able to be used for the treatment of patients with multidrug-resistant *M. tuberculosis*.

Acknowledgments

We thank S. Ehrt and A. Ding for helpful discussions, Y. Yamada and K. Takeda for technical assistance, and M. Kurata for secretarial assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Kaufmann, S. H. 2006. Tuberculosis: back on the immunologists' agenda. *Immunity* 24: 351–357.
2. North, R. J., and Y. J. Jung. 2004. Immunity to tuberculosis. *Annu. Rev. Immunol.* 22: 599–623.
3. Fremond, C. M., V. Yeremeev, D. M. Nicolle, M. Jacobs, V. F. Quesniaux, and B. Ryffel. 2004. Fatal *Mycobacterium tuberculosis* infection despite adaptive immune response in the absence of MyD88. *J. Clin. Invest.* 114: 1790–1799.
4. Quesniaux, V., C. Fremond, M. Jacobs, S. Parida, D. Nicolle, V. Yeremeev, F. Bihl, F. Erard, T. Botha, M. Drennan, et al. 2004. Toll-like receptor pathways in the immune responses to mycobacteria. *Microbes Infect.* 6: 946–959.
5. Gerritsen, J. 2000. Host defence mechanisms of the respiratory system. *Paediatr. Respir. Rev.* 1: 128–134.
6. Clauss, A., H. Lilja, and A. Lundwall. 2005. The evolution of a genetic locus encoding small serine proteinase inhibitors. *Biochem. Biophys. Res. Commun.* 333: 383–389.
7. Eisenberg, S. P., K. K. Hale, P. Heimdal, and R. C. Thompson. 1990. Location of the protease-inhibitory region of secretory leukocyte protease inhibitor. *J. Biol. Chem.* 265: 7976–7981.
8. Hagiwara, K., T. Kikuchi, Y. Endo, Huqun, K. Usui, M. Takahashi, N. Shibata, T. Kusakabe, H. Xin, S. Hoshi, et al. 2003. Mouse SWAM1 and SWAM2 are antibacterial proteins composed of a single whey acidic protein motif. *J. Immunol.* 170: 1973–1979.
9. Abe, T., N. Kobayashi, K. Yoshimura, B. C. Trapnell, H. Kim, R. C. Hubbard, M. T. Brewer, R. C. Thompson, and R. G. Crystal. 1991. Expression of the secretory leukoprotease inhibitor gene in epithelial cells. *J. Clin. Invest.* 87: 2207–2215.
10. Hiemstra, P. S., S. van Wetering, and J. Stolk. 1998. Neutrophil serine proteinases and defensins in chronic obstructive pulmonary disease: effects on pulmonary epithelium. *Eur. Respir. J.* 12: 1200–1208.
11. Schiessler, H., E. Fink, and H. Fritz. 1976. Acid-stable proteinase inhibitors from human seminal plasma. *Methods Enzymol.* 45: 847–859.
12. Vogelmeier, C., R. C. Hubbard, G. A. Fells, H. P. Schnebli, R. C. Thompson, H. Fritz, and R. G. Crystal. 1991. Anti-neutrophil elastase defense of the normal human respiratory epithelial surface provided by the secretory leukoprotease inhibitor. *J. Clin. Invest.* 87: 482–488.
13. Wingens, M., B. H. van Bergen, P. S. Hiemstra, J. F. Meis, I. M. van Vlijmen-Willems, P. L. Zeeuwen, J. Mulder, H. A. Kramps, F. van Ruissen, and J. Schalkwijk. 1998. Induction of SLPI (ALP/HUSI-I) in epidermal keratinocytes. *J. Invest. Dermatol.* 111: 996–1002.
14. Gauthier, F., U. Fryksmark, K. Ohlsson, and J. G. Bieth. 1982. Kinetics of the inhibition of leukocyte elastase by the bronchial inhibitor. *Biochim. Biophys. Acta* 700: 178–183.
15. Thompson, R. C., and K. Ohlsson. 1986. Isolation, properties, and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase. *Proc. Natl. Acad. Sci. USA* 83: 6692–6696.
16. Ashcroft, G. S., K. Lei, W. Jin, G. Longenecker, A. B. Kulkarni, T. Greenwell-Wild, H. Hale-Donze, G. McGrady, X. Y. Song, and S. M. Wahl. 2000. Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing. *Nat. Med.* 6: 1147–1153.

17. Zhu, J., C. Nathan, W. Jin, D. Sim, G. S. Ashcroft, S. M. Wahl, L. Lacomis, H. Erdjument-Bromage, P. Tempst, C. D. Wright, and A. Ding. 2002. Conversion of proepithelin to epithelins: roles of SLPI and elastase in host defense and wound repair. *Cell* 111: 867–878.
18. Hiemstra, P. S., R. J. Maassen, J. Stolk, R. Heinzel-Wieland, G. J. Steffens, and J. H. Dijkman. 1996. Antibacterial activity of antileukoprotease. *Infect. Immun.* 64: 4520–4524.
19. McNeely, T. B., D. C. Shugars, M. Rosendahl, C. Tucker, S. P. Eisenberg, and S. M. Wahl. 1997. Inhibition of human immunodeficiency virus type 1 infectivity by secretory leukocyte protease inhibitor occurs prior to viral reverse transcription. *Blood* 90: 1141–1149.
20. Tomee, J. F., P. S. Hiemstra, R. Heinzel-Wieland, and H. F. Kauffman. 1997. Antileukoprotease: an endogenous protein in the innate mucosal defense against fungi. *J. Infect. Dis.* 176: 740–747.
21. Simpson, A. J., A. I. Maxwell, J. R. Govan, C. Haslett, and J. M. Sallenave. 1999. Elafin (elastase-specific inhibitor) has anti-microbial activity against Gram-positive and Gram-negative respiratory pathogens. *FEBS Lett.* 452: 309–313.
22. Yenugu, S., R. T. Richardson, P. Sivashanmugam, Z. Wang, M. G. O'Rand, F. S. French, and S. H. Hall. 2004. Antimicrobial activity of human EPPIN, an androgen-regulated, sperm-bound protein with a whey acidic protein motif. *Biol. Reprod.* 71: 1484–1490.
23. Jin, F. Y., C. Nathan, D. Radzioch, and A. Ding. 1997. Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide. *Cell* 88: 417–426.
24. Taggart, C. C., S. A. Cryan, S. Weldon, A. Gibbons, C. M. Greene, E. Kelly, T. B. Low, S. J. O'Neill, and N. G. McElvaney. 2005. Secretory leucoprotease inhibitor binds to NF-κB binding sites in monocytes and inhibits p65 binding. *J. Exp. Med.* 202: 1659–1668.
25. Taggart, C. C., C. M. Greene, N. G. McElvaney, and S. O'Neill. 2002. Secretory leucoprotease inhibitor prevents lipopolysaccharide-induced IκBα degradation without affecting phosphorylation or ubiquitination. *J. Biol. Chem.* 277: 33648–33653.
26. Nakamura, A., Y. Mori, K. Hagiwara, T. Suzuki, T. Sakakibara, T. Kikuchi, T. Igarashi, M. Ebina, T. Abe, J. Miyazaki, et al. 2003. Increased susceptibility to LPS-induced endotoxin shock in secretory leukoprotease inhibitor (SLPI)-deficient mice. *J. Exp. Med.* 197: 669–674.
27. Ding, A., H. Yu, J. Yang, S. Shi, and S. Ehrt. 2005. Induction of macrophage-derived SLPI by *Mycobacterium tuberculosis* depends on TLR2 but not MyD88. *Immunology* 116: 381–389.
28. Doi, T., H. Yamada, T. Yajima, W. Wajjwalku, T. Hara, and Y. Yoshikai. 2007. H2–M3-restricted CD8$^+$ T cells induced by peptide-pulsed dendritic cells confer protection against *Mycobacterium tuberculosis*. *J. Immunol.* 178: 3806–3813.
29. deMello, D. E., S. Mahmoud, P. J. Padfield, and J. W. Hoffmann. 2000. Generation of an immortal differentiated lung type-II epithelial cell line from the adult H-2K^{b}tsA58 transgenic mouse. *In Vitro Cell. Dev. Biol. Anim.* 36: 374–382.
30. Aoki, K., S. Matsumoto, Y. Hirayama, T. Wada, Y. Ozeki, M. Niki, P. Domenech, K. Umemori, S. Yamamoto, A. Mineda, et al. 2004. Extracellular mycobacterial DNA-binding protein 1 participates in mycobacterium-lung epithelial cell interaction through hyaluronic acid. *J. Biol. Chem.* 279: 39798–39806.
31. Loh, B., C. Grant, and R. E. Hancock. 1984. Use of the fluorescent probe 1-*N*-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 26: 546–551.
32. Jat, P. S., M. D. Noble, P. Ataliotis, Y. Tanaka, N. Yannoutsos, L. Larsen, and D. Kioussis. 1991. Direct derivation of conditionally immortal cell lines from an H-2K^b-tsA58 transgenic mouse. *Proc. Natl. Acad. Sci. USA* 88: 5096–5100.
33. Whitehead, R. H., P. E. VanEeden, M. D. Noble, P. Ataliotis, and P. S. Jat. 1993. Establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2K^b-tsA58 transgenic mice. *Proc. Natl. Acad. Sci. USA* 90: 587–591.
34. Si-Tahar, M., D. Merlin, S. Sitaraman, and J. L. Madara. 2000. Constitutive and regulated secretion of secretory leukocyte proteinase inhibitor by human intestinal epithelial cells. *Gastroenterology* 118: 1061–1071.
35. Kirchhoff, C., I. Habben, R. Ivell, and N. Krull. 1991. A major human epididymis-specific cDNA encodes a protein with sequence homology to extracellular proteinase inhibitors. *Biol. Reprod.* 45: 350–357.
36. Miyakawa, Y., P. Ratnakar, A. G. Rao, M. L. Costello, O. Mathieu-Costello, R. I. Lehrer, and A. Catanzaro. 1996. In vitro activity of the antimicrobial peptides human and rabbit defensins and porcine leukocyte protegrin against *Mycobacterium tuberculosis*. *Infect. Immun.* 64: 926–932.
37. Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. *Nature* 415: 389–395.
38. Tanabe, H., X. Qu, C. S. Weeks, J. E. Cummings, S. Kolusheva, K. B. Walsh, R. Jelinek, T. K. Vanderlick, M. E. Selsted, and A. J. Ouellette. 2004. Structure-activity determinants in Paneth cell α-defensins: loss-of-function in mouse cryptdin-4 by charge-reversal at arginine residue positions. *J. Biol. Chem.* 279: 11976–11983.
39. Grutter, M. G., G. Fendrich, R. Huber, and W. Bode. 1988. The 2.5 A X-ray crystal structure of the acid-stable proteinase inhibitor from human mucous secretions analysed in its complex with bovine α-chymotrypsin. *EMBO J.* 7: 345–351.
40. Lin, C. C., and J. Y. Chang. 2006. Pathway of oxidative folding of secretory leucocyte protease inhibitor: an 8-disulfide protein exhibits a unique mechanism of folding. *Biochemistry* 45: 6231–6240.