Induction of macrophage-derived SLPI by *Mycobacterium tuberculosis* depends on TLR2 but not MyD88

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Summary

Macrophages respond to *Mycobacterium tuberculosis* by regulating expression of gene products that initiate a host innate response to this microorganism. In this study, we report that exposure of murine peritoneal macrophages to heat-killed *Mycobacterium tuberculosis* (HK-Mtb) led to an increase in secretory leucocyte protease inhibitor (SLPI) gene expression and protein secretion in a time- and dose-dependent manner. HK-Mtb-induced SLPI mRNA expression was sensitive neither to a protein synthesis inhibitor, cycloheximide, nor to an actin polymerization blocker, cytochalasin D. Treatment of macrophages with interferon (IFN)-γ inhibited HK-Mtb-induced SLPI expression. RAW264·7 cells stably expressing SLPI produced a reduced level of tumour necrosis factor (TNF) in response to HK-Mtb as compared with mock transfectants. Aerosol infection of mice with live *M. tuberculosis* resulted in an induction of SLPI gene expression in infected lungs. Macrophages from Toll-like receptor 4 (TLR4)$^{-/-}$ or MyD88$^{-/-}$ mice responded to *M. tuberculosis* similarly to wild-type macrophages by exhibiting increased SLPI expression. In contrast, macrophages from TLR2$^{-/-}$ mice were incapable of inducing SLPI in response to *M. tuberculosis*. This induction signifies the presence of a TLR2-dependent but MyD88-independent *M. tuberculosis* signalling pathway, suggesting involvement of adaptor protein(s) other than MyD88 in *M. tuberculosis*-mediated induction of SLPI.

Keywords: rodent; macrophage; mycobacteria; signal transduction; protease inhibitor

Introduction

Toll-like receptors (TLRs) are a family of evolutionarily conserved surface molecules that contain extracellular leucine-rich repeats and an intracellular Toll/interleukin (IL)-1 receptor homology (TIR) domain.[1–3] TLRs play a crucial role in the host innate recognition of and response to a variety of microbial pathogens. The best studied TLR agonist is the lipopolysaccharide (LPS) of Gram-negative bacteria. LPS can be recognized by TLR4 or TLR2, depending on its bacterial origin. The immediate downstream signalling event following LPS/TLR4 interaction is the recruitment of a family of adaptor proteins, including MyD88, TIR domain-containing adapter protein (TIRAP), TIR-containing adapter inducing interferon (IFN)-β (TRIF or Lps2) and TRIF-related adapter molecule (TRAM or TIRP). In contrast, other TLRs except TLR2 and TLR3 appear to use only MyD88 to transduce signals. Activation

of TLR2 utilizes TIRAP in addition to MyD88,[4,5] while activation of TLR3 by double-stranded RNA only utilizes TRIF.[6,7]

Mycobacterium tuberculosis, the causative agent of pulmonary tuberculosis, has been shown to activate macrophages via TLR2 and TLR4.[8–11] *M. tuberculosis*-mediated macrophage activation is important to allow the host to restrain *M. tuberculosis* infection[12] and is accompanied by induction of a wide spectrum of bioactive cellular products.[13] Microarray analysis with MyD88$^{-/-}$ cells, however, revealed that the majority of *M. tuberculosis*-induced gene expression in mouse bone marrow-derived macrophages is independent of MyD88.[14] It is not known what surface receptors initiate the MyD88-independent signalling pathways triggered by *M. tuberculosis*.

Secretory leucocyte protease inhibitor (SLPI), an 11·7-kDa cysteine-rich secretory protein, was originally isolated from human saliva[15] and subsequently found in

many other body fluids, including bronchial mucus[16] and plasma.[17] Tracheal, bronchial and type II alveolar cells as well as macrophages and neutrophils are rich sources of SLPI in the lung. Administration of exogenous recombinant SLPI via intratracheal instillation or aerosol inhalation has been shown to control lung inflammation induced by immune complex deposition,[18] neutrophil elastase[19] or LPS.[20] The level of endogenous SLPI during infection is under strict control. Macrophages produced an elevated level of SLPI in response to constituents of Gram-negative and Gram-positive bacteria such as LPS[17] and lipoteichoic acid,[21] to cytokines IL-6 and IL-10[21] and to apoptotic cells.[22] As the agent of pulmonary tuberculosis, *M. tuberculosis* infects the lungs of the host. It is not known how *M. tuberculosis* infection affects endogenous SLPI levels, nor how endogenous SLPI levels affect host responses to *M. tuberculosis*. The present study was designed to characterize macrophage responses to *M. tuberculosis* in terms of SLPI expression. During the course of study, we discovered that *M. tuberculosis*-mediated SLPI induction utilizes a novel *M. tuberculosis* signalling pathway. This pathway depends on TLR2 but not MyD88.

Materials and methods

Mice

Adult TLR2$^{-/-}$ TLR4$^{-/-}$ MyD88$^{-/-}$ mice were generated by Dr S. Akira's laboratory (Osaka University, Osaka, Japan) as previously described.[23–25] These mice had been backcrossed six times to the C57BL/6 background. TRIF mutant mice were provided by Dr B. Beutler (the Scripps Institute, La Jolla, CA) and TIRAP$^{-/-}$ mice by Dr R. Medzhitov (Yale University School of Medicine, New Haven, CT). Age-matched wild-type littermates were bred in the Animal Research Center at the Weill Medical College of Cornell University (New York, NY). Wild-type C57BL/6 mice were purchased from the Charles River Breeding Laboratories (Wilmington, MA).

Cells

Primary mouse peritoneal macrophages were collected from the peritoneal cavity 4 days after intraperitoneal injection with 2 ml of 4% Brewer's thioglycollate broth (Difco, Detroit, MI). Bone marrow-derived macrophages were isolated and differentiated in culture as previously described.[14] The RAW264·7 mouse macrophage cell line was from the American Type Culture Collection (ATCC; Manassas, VA). RAW cells stably expressing high levels of SLPI were generated as previously described.[22] Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine,

200 units/ml penicillin and 200 µg/ml streptomycin (complete medium) at 37° in 5% CO_2/95% air. Complete culture medium was routinely monitored for LPS contamination and found to contain < 25 pg LPS/ml, as determined by the limulus amebocyte lysate assay (Bio-Whittaker, Walkersville, MD).

Detection of SLPI protein in culture supernatants or cell lysates

The proteins in serum-free culture supernatants from 10^7 cells were precipitated with ice-cold 10% trichloroacetic acid (TCA) for 30 min. The precipitates were rinsed twice in acetone and boiled for 5 min in Laemmli buffer. For cell lysates, 2×10^6 cells were collected and boiled directly in Laemmli sample buffer. Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Schleicher and Shuell, Inc., Keene, NH). The membrane was blocked with 5% milk and blotted with an anti-mouse SLPI IgG (1 : 5000)[26] followed by a donkey anti-rabbit IgG coupled to horseradish peroxidase (HRP). The bound antibody was detected by an enhanced chemiluminescent substrate for HRP (Pierce, St Louis, MO).

Mouse infections

Mice were infected with logarithmic growth phase cultures of *M. tuberculosis* by aerosol using a Glas-Col Inhalation Exposure System (Glas-col Inc., Terre Haute, IN). Animals were exposed for 30 min to an aerosol produced by nebulizing 5 ml of a bacterial suspension in phosphate-buffered saline at a concentration of ∼2×10^7 bacilli/ml. This resulted in an inoculum size of 50–70 colony-forming units (CFU) per lung as determined by plating homogenized lungs onto enriched 7H11 plates 24 hr post-infection.

Infection of macrophages

Thioglycollate-elicited macrophages were cultured in 100-mm-diameter culture dishes (10^7 cells/dish) without antibiotics for 24 hr and then infected with live or heat-killed (HK) *M. tuberculosis* strain 1254 from early log-phase cultures.[14]

Northern blot

Total RNA (20 µg/lane) was electrophoresed on a 1% agarose gel with 20 mM 3-[N-morpholino] propane-sulphonic acid, pH 7·0, 50 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA) (1 × MOPS) and 2% formaldehyde, and equal loading was confirmed by ethidium bromide staining. RNA was transferred in 20 × saline sodium citrate (SSC) onto a nylon membrane

(NEN; Research Products, Boston, MA). The membrane was hybridized for 18 hr at 42° with labelled probe [10^6 counts per minute (c.p.m.)/ml] in 5 × SSC, 5 × Denhart, 50% formamide and 1% SDS. Membranes were then washed twice with 1 × SSC and 0·1% SDS (10 min; 25°) and with 0·25 × SSC and 0·1% SDS (10 min, 55°) before exposure to X-Omat AR film (Kodak, Rochester, NY). Control probe for β-actin cDNA was amplified using the manufacturer's templates and amplimers (Clontech, Palo Alto, CA). The probes were radiolabelled with the Priming-a-gene kit from Promega (Madison, WI).

Real-time quantitative reverse transcriptase–polymerase chain reaction (qPCR)

The 5′, 3′ nuclease activity of Taq polymerase was used to detect PCR-amplified nucleic acids. Standard curves were generated for each gene product using a plasmid dilution series containing the target sequences. Probes were synthesized by Biosearch Technologies (Novato, CA) and labelled with the reporter dye fluorescent amidites (FAM) at the 5′ end and the quencher Black Hole Quencher (BHQ) at the 3′ end. Primer and probe sequences for SLPI were: qPCR primer (forward) 5′-d(GCTGTGAGGG TATATGTGGGAAA)-3′, qPCR primer (reverse) 5′-d(CG CCAATGTCAGGGATCAG)-3′, and qPCR probe 5′-FAMd(TCTGCCTGCCCCCGATGTGAG)BHQ-3′. Primer and probe sequences for GAPDH were: qPCR primer (forward) 5′-d(GGGAAGCCCATCACCATCTT)-3′, qPCR primer (reverse) 5′-d(ACATACTCAGCACCGGCCTC)-3′, and qPCR probe 5′-FAMd(AGCGAGACCCCACTAACA TCAAATGGG)BHQ-3′. RNA (100 ng) was transcribed into cDNA with gene-specific primers in 20 μl using 50 U MuLV reverse transcriptase (Perkin Elmer, Wellesley, MA). cDNA was diluted to 100 μl. PCR was performed in a volume of 15 μl on the ABI PRISM 7900HT sequence detection system (Perkin Elmer).

Enzyme-linked immunosorbent assay (ELISA)

Cultured supernatants were collected and tested for tumour necrosis factor (TNF) contents using a Duoset ELISA (R & D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Reagents and supplies

M. tuberculosis strain 1254, a low-passage clinical isolate, was from American Type Culture Collection 51910. Heat-killed *M. tuberculosis* (HK-Mtb) was prepared by incubating *M. tuberculosis* 1254 at 80° for 8 hr. LPS was prepared by phenol extraction according to the procedure of Qureshi et al.[27] from *Escherichia coli* 0111:B4 LPS (Sigma, St Louis, MO). Peptidoglycan from *Staphylococcus aureus* was purchased from Fluka (Milwaukee, WI). Cyclohexi-

mide was from Sigma. Purified recombinant mouse IFN-γ (protein concentration 1 mg/ml; specific activity 10^7 U/mg; LPS content < 52 pg/ml) was from Genentech (South San Francisco, CA). Oligonucleotide primers were from Invitrogen (Carlsbad, CA). G418 was from Gibco Life Technologies (Grand Island, NY); AmpliTaq DNA polymerase, dNTPs, and PCR buffer solutions and [$α$-^{32}P]-deoxycytidine 5′-triphosphate (dCTP) were from Perkin Elmer Cetus (Foster City, CA). Plasmid DNA preparation columns were from Qiagen (Chasworth, CA).

Statistical analysis

The Student's *t*-test was used for analysis of the real-time PCR and ELISA.

Results

Heat-killed *M. tuberculosis* triggers SLPI release from macrophages *in vitro*

Mouse peritoneal macrophages were incubated for 90 min with HK-Mtb at a multiplicity of infection (MOI) from 1 to 8 in serum-free medium for 24 hr at 37°. Culture medium was collected and SLPI content evaluated by western blot analysis after TCA precipitation (Fig. 1a). SLPI was secreted by macrophages exposed to HK-Mtb in a dose-dependent manner. Similar results were obtained in experiments using RAW264·7 cells (data not shown). Kinetics study showed that the secretion of SLPI from primary cells increased as early as 12 hr after exposure of macrophages to HK-Mtb, and continued to increase up to 48 hr (Fig. 1b).
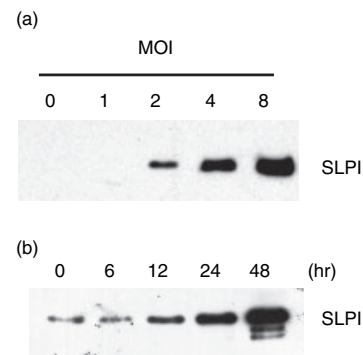

Figure 1. Secretion of secretory leucocyte protease inhibitor (SLPI) by macrophages in response to heat-killed *Mycobacterium tuberculosis* (HK-Mtb). (a) Dose curve. Peritoneal macrophages (10^7) were treated with HK-Mtb [multiplicity of infection (MOI) from 1 to 8] for 24 hr. Protein samples from cultured supernatants were trichloroacetic acid (TCA)-precipitated, electrophoresed, and immunoblotted with anti-SLPI antibody. (b) Time–course. Secreted SLPIs from macrophages (10^7) treated with HK-Mtb (MOI = 4) for the indicated times were prepared and analysed as in (a).
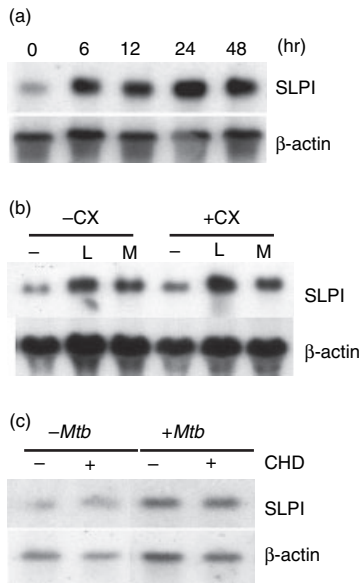
Figure 2. Heat-killed *Mycobacterium tuberculosis* (HK-Mtb)-induced secretory leucocyte protease inhibitor (SLPI) expression and the effect of cycloheximide or cytochalasin D. (a) Peritoneal macrophages (10^7) were incubated alone or with HK-Mb [multiplicity of infection (MOI) = 4] for the indicated times. Total RNA was prepared, electrophoresed and hybridized with SLPI cDNA probe. The membrane was rehybridized with β-actin probe as a loading control. (b, c) Macrophages were pretreated with 10 μg/ml cycloheximide (CX) or 5 μg/ml of cytochalasin D (CHD) for 30 min, then incubated with 100 ng/ml lipopolysaccharide (L) or HK-Mtb (M, MOI = 4) for an additional 6 hr. Northern blots were prepared and analysed as in (a).

Induction of SLPI expression by exposure of macrophages to HK-Mtb

To test whether HK-Mtb-induced release of SLPI is caused by the triggering of preformed intracellular SLPI or by the induction of SLPI gene expression, we compared SLPI mRNA levels in macrophages in the presence and absence of HK-Mtb. HK-Mtb stimulated an increase in SLPI expression in a time-dependent fashion (Fig. 2a). Induction of SLPI by HK-Mtb was not detectable at 2 hr (not shown) but was readily detectable at 6 hr, and remained high throughout the study (up to 48 hr). To address the possibility that the SLPI gene might be induced indirectly by a HK-Mtb-inducible product, we examined the effect of the protein synthesis inhibitor cycloheximide. At 10 μg/ml, cycloheximide inhibited protein synthesis in primary macrophages by 95%,[21] but the increased expression of SLPI by HK-Mtb, like that induced by LPS,[21] was not inhibited (Fig. 2b). Next we tested whether phagocytosis of HK-Mtb was necessary for SLPI induction. Primary macrophages were pretreated with the actin polymerization blocker cytochalasin D for 30 min before the addition of HK-Mtb, and SLPI

expression was analysed using northern blots. Although cytochalasin D blocked phagocytosis of HK-Mtb by macrophages (not shown), HK-Mtb-induced SLPI expression was not affected (Fig. 2c).

Effects of IFN-γ on SLPI expression

As the major activator of macrophages, IFN-γ is capable of inducing macrophages to produce a similar spectrum of immune mediators to those induced by many microbial products. The cardinal role of IFN-γ in host defence against *M. tuberculosis* infection is highlighted by the early onset of systemic *M. tuberculosis* infection and the early death of IFN-γ knockout mice.[28] To test how IFN-γ affects SLPI induction by *M. tuberculosis*, peritoneal macrophages were incubated with LPS from *E. coli*, peptidoglycan from *S. aureus* and HK-Mtb in the presence or absence of IFN-γ for 24 hr. SLPI expression was induced by these three microbial products but not by IFN-γ alone. Co-incubation with IFN-γ suppressed SLPI induction, but enhanced TNF induction by each of these stimuli (Fig. 3). In a separate experiment, IFN-γ was preincubated with peritoneal macrophages for 24 hr before addition of HK-Mtb. Suppression of HK-Mtb-induced SLPI was observed again, regardless of whether IFN-γ was added before or together with HK-Mtb (not shown).

HK-Mtb-induced SLPI expression depends on TLR2, but not TLR4 or MyD88

Mycobacterium tuberculosis is known to express both TLR2 and TLR4 agonists, which are usually presumed to
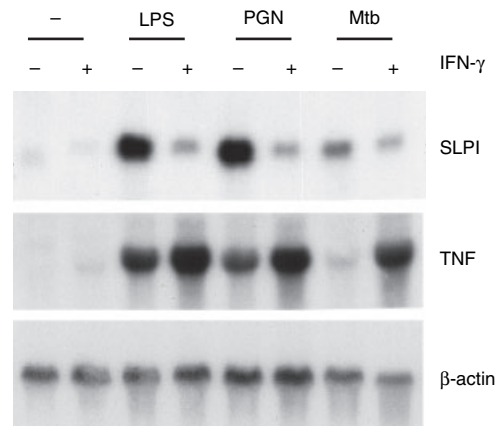

Figure 3. Effect of interferon (IFN)-γ on heat-killed *Mycobacterium tuberculosis* (HK-Mtb)-induced secretory leucocyte protease inhibitor (SLPI) expression. Peritoneal macrophages (10^7) were incubated with 100 ng/ml lipopolysaccharide (LPS), or 10 μg/ml peptidoglycan (PGN), or 4×10^7 HK-Mtb in the presence or absence of 100 U/ml of IFN-γ for 24 hr. SLPI and tumour necrosis factor (TNF) mRNA levels were analysed by northern blots, controlled and expressed as in Fig. 2.
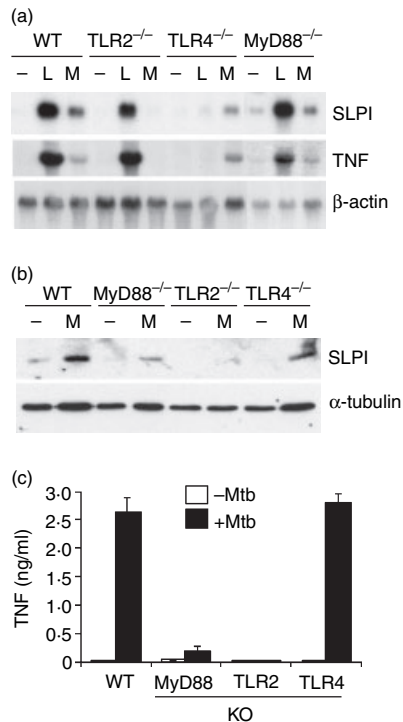
Figure 4. Induction of secretory leucocyte protease inhibitor (SLPI) expression by *Mycobacterium tuberculosis* depends on Toll-like receptor 2 (TLR2) but not TLR4 or MyD88. Peritoneal macrophages from wild-type (WT), TLR2$^{-/-}$, TLR4$^{-/-}$ or MyD88$^{-/-}$ mice were incubated (a–c) with either heat-killed *Mycobacterium tuberculosis* (HK-Mtb) [M, multiplicity of infection (MOI) = 4] or lipopolysaccharide (L, 100 ng/ml) for 24 hr. (a) The expression of the secretory leucocyte protease inhibitor (SLPI) and tumour necrosis factor (TNF) mRNA was assessed by northern blot analysis as described in Fig. 2. (b) SLPI protein expression in the lysate was shown via western blot using tubulin as a loading control. (c) TNF protein in the culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA). KO, knock-out mice.

signal via MyD88. To investigate the role of these molecules in SLPI induction by HK-Mtb, macrophages from wild-type, TLR2$^{-/-}$, TLR4$^{-/-}$ or MyD88$^{-/-}$ mice were tested for SLPI induction after exposure to HK-Mtb. Figure 4(a) shows that SLPI mRNA induction by LPS and HK-Mtb was diminished in macrophages from TLR4$^{-/-}$ and TLR2$^{-/-}$ mice, respectively. Macrophages from MyD88$^{-/-}$ mice, however, responded to both stimuli normally. In the same experiment, the pattern of TNF mRNA induction by HK-Mtb resembled that of SLPI induction, but TNF mRNA induction by LPS was reduced in MyD88$^{-/-}$ cells. Induction of SLPI and TNF at the protein level was also examined. As shown in Figs 4(b) and (c), both SLPI and TNF proteins were detected from HK-Mtb-treated macrophages of wild-type and TLR4$^{-/-}$ mice, but not from those of TLR2$^{-/-}$ mice. Macrophages from MyD88$^{-/-}$ mice responded to HK-Mtb by producing little TNF but readily detectable
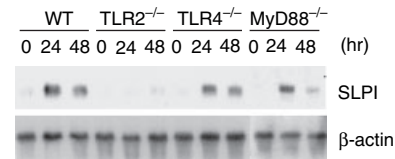

Figure 5. Induction of secretory leucocyte protease inhibitor (SLPI) expression by live *Mycobacterium tuberculosis* depends on Toll-like receptor 2 (TLR2) but not TLR4 or MyD88. Peritoneal macrophages from wild-type (WT), TLR2$^{-/-}$, TLR4$^{-/-}$ or MyD88$^{-/-}$ mice were incubated with live *M. tuberculosis* [multiplicity of infection (MOI) = 4] for the time indicated. The expression of the SLPI mRNA was assessed by northern blot analysis as described in Fig. 2.

levels of SLPI, albeit at a reduced level compared with wild-type cells.

To test whether live *M. tuberculosis* acts in a similar manner, macrophages from wild-type, TLR2$^{-/-}$, TLR4$^{-/-}$ or MyD88$^{-/-}$ mice were incubated with live *M. tuberculosis* (MOI = 4) for 24 or 48 hr and SLPI expression was examined. As shown in Fig. 5, live *M. tuberculosis* induced SLPI expression in an identical fashion to HK-Mtb. Thus, LPS induced SLPI expression via a TLR4-dependent but MyD88-independent mechanism, and *M. tuberculosis* induced SLPI expression via a TLR2-dependent but MyD88-independent mechanism. The same induction pattern was observed using bone marrow-derived macrophages (not shown). In addition, macrophages from TIRAP-null and TRIF-mutated mice responded to live *M. tuberculosis* by expressing elevated levels of the SLPI gene (not shown), suggesting that none of these MyD88-like proteins has a role in mediating TLR2-dependent induction of SLPI by *M. tuberculosis*.

Infection of mice with *M. tuberculosis* induces SLPI expression in the lung

The results reported so far were from *in vitro* studies. To see whether SLPI can be induced by *M. tuberculosis in vivo*, we employed an established mouse model in which a low dose of *M. tuberculosis* (100 CFU/mouse) is delivered to mice via aerosol infection. This infection leads to a bacterial load of approximately $1·4 \times 10^6$ bacteria per lung at 21 days post-infection, as determined by CFU on 7H11 agar plates. SLPI expression in infected lungs was determined at day 10, 21, 56 or 71 post-infection. Induction of SLPI was detected in the early (10 and 21 days) but not late (56 and 71 days) time-points after aerosol infection (Fig. 6).

SLPI overexpression led to suppression of TNF production in response to HK-Mtb

Forced expression of SLPI in macrophage cell lines induced a LPS-hyporesponsive state.[26,29] To determine
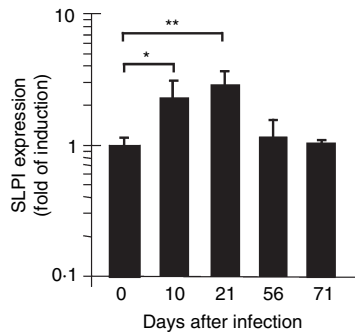
Figure 6. Secretory leucocyte protease inhibitor (SLPI) induction in the lungs after low-dose *Mycobacterium tuberculosis* aerosol infection. RNA was prepared from lungs of mice that were infected with 100 colony-forming units (CFU) of *M. tuberculosis* for different times up to 71 days. Real-time quantitative reverse transcriptase–polymerase chain reaction (qPCR) was performed as described in the 'Materials and methods' section. The results are mean ± standard deviation of triplicate samples from one of two experiments. The data were analysed using Student's *t*-test (*$P = 0.065$; **$P = 0.041$).

whether SLPI expression has any impact on HK-Mtb-induced TNF-α production in macrophages, we compared HK-Mtb-induced TNF between two stable transfectants of RAW164·7 cells expressing either SLPI (RAW-SLPI) or vector only (RAW-mock). These cells lines have been characterized.[22] SLPI-expressing cells and mock transfectants were incubated with HK-Mtb at MOIs from 1 to 8 for 16 hr. TNF levels in the media were assessed by ELISA. As shown in Fig. 7, TNF production by SLPI-
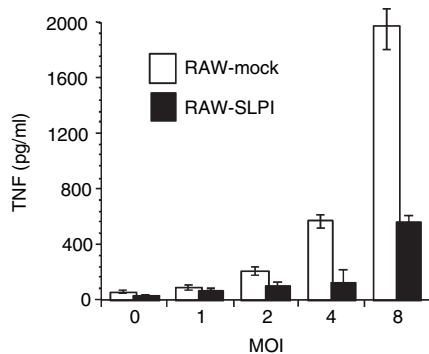

Figure 7. Secretory leucocyte protease inhibitor (SLPI) expression in macrophages leads to a suppression of tumour necrosis factor (TNF) production in response to *Mycobacterium tuberculosis*. RAW 264·7 cells stably transfected with SLPI (■) or vector only (□) were treated with heat-killed *Mycobacterium tuberculosis* (HK-Mtb) at the indicated multiplicity of infection (MOI) for 16 hr. Conditioned media were collected for TNF determination by enzyme-linked immunosorbent assay (ELISA). Results are mean ± standard deviation of triplicates from one of four similar experiments. Student's *t*-test analysis shows that the differences between TNF production between two cell lines are significant at MOI 4 and 8 ($P < 0.05$, Student's *t*-test).

expressing cells was greatly reduced in response to HK-Mtb, compared with that by mock transfectants.

Discussion

The secretory leucocyte protease inhibitor (SLPI) has a multifaceted role in host inflammatory responses to infections.[30] It inhibits leucocyte-derived proteases,[31] exerts antimicrobial[32] and antiviral activity,[33] and suppresses the ongoing secretion of inflammatory mediators by macrophages.[26,29,34] Because of its potent anti-inflammatory action, SLPI has been used as an effective therapy for disorders characterized by an increased inflammation, especially in the lung.[35,36] We report here that both heat-killed and live *M. tuberculosis* induced SLPI production by mouse macrophages *in vitro*. *M. tuberculosis*-induced SLPI expression probably resulted from the direct recognition and ligation of macrophage surface receptor(s) by *M. tuberculosis*, as this induction was not blocked by either a protein synthesis inhibitor or an actin polymerization inhibitor. Aerosol infection of mice with live *M. tuberculosis* also resulted in elevated SLPI expression in infected lungs. Thus, live and heat-killed *M. tuberculosis* join a long list of agents that are capable of inducing SLPI in macrophages.[17,21,22,37]

Although SLPI was reported to possess bactericidal activity against *E. coli* and *S. aureus in vitro*, we found that recombinant SLPI alone would not kill *M. tuberculosis in vitro*. The time–course of the slight increase of SLPI expression after *M. tuberculosis* infection *in vivo*, observed in this study, coincided with the development of inflammation in infected lungs. Because SLPI is a potent leucocyte protease inhibitor, and macrophages expressing a high level of SLPI tend to be less responsive to many microbial stimuli, including *M. tuberculosis*, it is possible that enhanced SLPI expression may help to control inflammation-associated tissue damage during *M. tuberculosis* infection.

Surprisingly, we found that *M. tuberculosis* induced SLPI expression in macrophages via a novel pathway that is dependent on TLR2 but not on MyD88. This conclusion was based on the fact that macrophages from MyD88 knockout mice responded to *M. tuberculosis* by inducing increased expression of SLPI, while macrophages from TLR2 knockout mice could not induce SLPI in response to *M. tuberculosis*. SLPI protein production was diminished in MyD88$^{-/-}$ cells compared with that in wild-type cells, whereas no difference was observed at the mRNA level, suggesting a role for MyD88 in post-transcriptional control of SLPI. However, induction of SLPI mRNA and protein was completely abolished in TLR2$^{-/-}$ cells, demonstrating that TLR2 is essential for transcriptional induction of SLPI. The phenotypes of the knockout mice were confirmed by their defects in inducing TNF in response to selective TLR ligands: TLR2-null cells did not

respond to Pam3, TLR4-null cells did not respond to LPS, and MyD88-null cells responded to neither stimulus (data not shown). TLR2 and TLR4 have been implicated in the recognition and signalling of *M. tuberculosis* in macrophages.[38] Early evidence that *M. tuberculosis* might act via TLR2 came from the studies using forced gene expression systems.[39] Brightbill *et al.* showed that stable expression of TLR2, but not its inactive mutant, in HEK293 cells conferred activation of a nuclear factor (NF)-κB-driven reporter gene by a 19-kDa lipoprotein from mycobacterial cell walls.[39] Underhill and his colleagues[40] demonstrated that mycobacterial cell wall components, including lipoarabinomannan, mycolylarabinogalactan–peptidoglycan complex, and *M. tuberculosis* total lipids, induced TNF in mouse RAW264·7 cells. Transient transfection of dominant negative TLR2 suppressed this response. Similar conclusions regarding the role of TLR2 were reached by Means *et al.* using Chinese hamster ovary cells as recipients.[41,42] These authors showed that, in addition to TLR2, TLR4 also mediated signalling of a heat-sensitive mycobacterial factor in CHO cells expressing CD14.[42]

The role of MyD88 in the host immune response against *M. tuberculosis* is controversial. On one hand, MyD88 has a cardinal role in innate immune responses utilizing almost all TLRs as well as IL-1R and IL-18R.[43] If TLR2 and TLR4 are responsible for *M. tuberculosis*-mediated cellular events in macrophages, MyD88 would be expected to be important for *M. tuberculosis*-mediated signals. Indeed, dominant negative MyD88 was shown to suppress the macrophage response to certain *M. tuberculosis* products.[40] On the other hand, induction of nitric oxide by viable *M. tuberculosis* bacilli was found in the absence of TLR2, TLR4 and MyD88.[8] These authors concluded that, while the TLR-dependent pathway depended on MyD88, the non-TLR-dependent pathway did not. In a transcriptome-wide analysis of *M. tuberculosis*-inducible genes in bone marrow-derived macrophages, the majority of *M. tuberculosis*-induced genes could be induced in the absence of MyD88.[14] In a follow-up study, most of these MyD88-independent genes were found also to be induced in macrophages from TLR2, TLR4 double-deficient mice (Shi *et al.*, unpublished observation). However, SLPI was regulated in a TLR2-dependent, MyD88-independent manner, as also demonstrated in this work.

Our finding suggests the presence of a MyD88-like adaptor protein(s) in TLR2-mediated signalling that functions independently of MyD88. Three MyD88-like proteins have been identified and implicated in LPS responses via TLR4. These include TIRAP/Mal,[4,44] TRIF/Lps2[6,45] and TRAM/TIRP.[46,47] TIRAP/Mal was found to be shared by TLR2 and TLR4. TIRAP/Mal-deficient cells responded to LPS in an identical fashion to MyD88-deficient cells, and thus did not function independently from MyD88. The adaptors TRIF/Lps2 and TRAM/TIRP do function in the absence of MyD88, but they are shared by TLR3 and TLR4 in response to double-stranded RNA and LPS, but not by TLR2 in response to peptidoglycan.[6,45] We found that macrophages from TIRAP knockout or TRIF-mutated mice were still capable of inducing SLPI after *M. tuberculosis* exposure, suggesting that these molecules are unlikely to be the adaptors that mediate TLR2-dependent SLPI induction by *M. tuberculosis*. Tests in TRAM knockout mice should help clarify whether TRAM or an unidentified adaptor is necessary for *M. tuberculosis*-induced SLPI expression as well as for TLR2-mediated signalling in general.

Acknowledgements

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