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Increased Susceptibility to LPS-induced Endotoxin Shock in Secretory Leukoprotease Inhibitor (SLPI)-deficient Mice

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The Journal of Experimental Medicine

Abstract

Secretory leukoprotease inhibitor (SLPI) protects tissue against the destructive action of neutrophil elastase at the site of inflammation. Recent studies on new functions of SLPI have demonstrated that SLPI may play a larger role in innate immunity than merely as a protease inhibitor. To clarify the functions of SLPI in bacterial infections, we generated SLPI-deficient mice ($SLPI^{-/-}$ mice) and analyzed their response to experimental endotoxin shock induced by lipopolysaccharide (LPS). $SLPI^{-/-}$ mice showed a higher mortality from endotoxin shock than did wild type mice. This may be explained in part by our observation that $SLPI^{-/-}$ macrophages show higher interleukin 6 and high-mobility group (HMG)-1 production and nuclear factor κB activities after LPS treatment than do $SLPI^{+/+}$ macrophages. SLPI also affects B cell function. $SLPI^{-/-}$ B cells show more proliferation and IgM production after LPS treatment than $SLPI^{+/+}$ B cells. Our results suggest that SLPI attenuates excessive inflammatory responses and thus assures balanced functioning of innate immunity.

Key words: endotoxin shock • innate immunity • LPS • NF-κB • SLPI

Introduction

Bacterial infection can evokes endotoxin shock characterized by fever, myocardial dysfunction, acute respiratory failure, and multiple organ failure, resulting in a high mortality rate. LPS, a major component of the outer membrane of Gram-negative bacteria, is one of the major toxins that initiate the cascade of pathophysiological reactions called endotoxin shock. In the bloodstream LPS is carried by the serum protein LPS-binding protein (LBP). On the surface of neutrophils, monocytes, lymphocytes, and macrophages (1, 2), LPS binds to CD14 and Toll-like receptor 4 (TLR4),* triggering the production of inflammatory cytokines and bioactive molecules which include TNF-α, IL-1 and IL-6, proteases, and NO (1, 2). Excessive production of cytokines is considered to be crucial to the initiation of the endotoxic shock cascade (1, 2). Neutrophil elastase and cathepsin G–deficient mice have been shown to be resistant to LPS-induced endotoxin shock (3), thus demonstrating that both cytokines and proteases define the susceptibility to endotoxin shock.

Secretory leukoprotease inhibitor (SLPI) is an 11.7 kD (107 amino acid), nonglycosylated, single-chain serine protease inhibitor (4). SLPI is produced by secretory cells in respiratory, genital and lacrimal glands, and by inflammatory cells that include macrophages, neutrophils, and B cells (5–8). SLPI inhibits several serine proteases. Examples are elastase and cathepsin G secreted from neutrophils, trypsin and chymotyrpsin from pancreatic acinar cells, and chymase and tryptase from mast cells (4). Therefore, a major physiological role of SLPI is considered to be the protection of tissue from these proteases at sites of inflammation (4). However, recent studies have demonstrated that SLPI functions as more than just a protease inhibitor. SLPI suppresses bacterial growth (9), inhibits infection of lymphocytes by human immunodeficiency virus-I (HIV-Ip; reference 10), ameliorates bacterial arthritis (11), and decreases production

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**Abbreviations used in this paper:* CLP, cecal ligation and puncture; ES, embryonic stem; HMG, high-mobility group; SLPI, secretory leukoprotease inhibitor; TLR, Toll-like receptor.

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of prostaglandin (PG)E2 and matrix metalloproteinases (MMP)-1 and 9 (12). SLPI is involved in normal cutaneous wound healing, a result previously shown in a study on SLPI$^{-/-}$ mice (13, 14). Moreover, SLPI modifies macrophage functions in mice, as shown by the fact that ectopic expression of SLPI cDNA in macrophages increases their resistance to LPS, and that SLPI suppresses macrophage response to LPS (7). Consistently, serum SLPI levels are elevated in endotoxin shock in human (15). These observations strongly suggest that SLPI is an important participant in innate immunity where it acts as an antiinflammatory molecule.

To clarify the function of SLPI in innate immunity, we generated a mouse strain lacking SLPI by gene targeting in embryonic stem cells and analyzed their response to experimental endotoxic shock induced by LPS. As expected, SLPI$^{-/-}$ mice were highly sensitive to LPS or cecal ligation and puncture (CLP)-induced sepsis compared with SLPI$^{+/+}$ mice. Macrophages lacking SLPI were also highly responsive to LPS with increased IL-6 and HMG (high-mobility group)-1 production and nuclear factor (NF)-κB activation. These results indicated that endogenous SLPI inhibited the signaling pathways though LPS-CD14:TLR4 resulting in the protective function upon septic shock.

Materials and Methods

Generation of SLPI$^{-/-}$ Mice. The SLPI genomic DNA was isolated from the 129/Sv mouse genomic library constructed into lambda Fix II (Stratagene). The insert was subcloned into pBluescript SK(-) vector (Stratagene), and confirmed by restriction enzyme mapping and DNA sequencing. A targeting vector was designed to replace all four exons of the SLPI gene with a PGK promoter driven NEO (neomycin resistance gene) expression cassette. The targeting vector was electroporated into E14.1 embryonic stem (ES) cells and selected with G418. Homologous recombinants were identified by Southern hybridization using a genomic probe located on the 3′ side of the SLPI gene: Southern hybridization detected a 5.0 kb StuI fragment of the endogenous SLPI allele and a 4.0 kb fragment of the targeted allele. The identified targeted ES clones were microinjected into the blastocysts of C57BL/6 mice. Chimeric mice were crossed with C57BL/6 females to generate heterozygous mice. Heterozygous mice were intercrossed to obtain homozygous mice. SLPI$^{-/-}$ mice and their wild-type (SLPI$^{+/+}$) littermates were used in this study. These mice were kept and bred in the Animal Unit of The Institute of Development, Aging and Cancer, a facility which is environmentally controlled and specific pathogen-free.

Southern Blot Analysis and PCR Genotyping. For Southern blot analysis, DNA was isolated from G418-resistant ES cells or from mouse liver and digested with StuI. Hybridization was performed with ^{32}P (Amersham Biosciences)-radiolabeled 5′ or 3′ probes indicated in the figure. For PCR genotyping, DNA was isolated from mouse-tail biopsies. PCR primers used were p38F1 (forward: 5′-CATGTGAACACTTCAGAAGAGAAGG-3′) and PGKR1 (reverse: 5′-GCTACTTCCATTTGTCACGTCCTGC-3′) to detect the knockout allele, or p38F1 and SLPIR1 (reverse: 5′-GTGAGATGCTGAGAACTAAAGCCAG-3′) to detect the wild-type allele. Amplification was performed using rTth DNA polymerase (PerkinElmer) by 40 cycles of 40 s at 94°C, 30 s at 55°C, and 4 min at 68°C. For RT-PCR, total RNA was isolated from macrophages by RNeasy Mini Kit (QIAGEN). Primers used were forward 5′-ATGAAGTCCTGCGGCCTTTT-3′ and reverse 5′-GCATAGAGAAATGAATGCGT-3′ for SLPI, and forward 5′-CTACAATGAGCTGCGTGTGG-3′ and reverse 5′-AGGAAGGCTGAAGAGTGC-3′ for β-actin. Amplification was performed by OneStep RT-PCR kit (QIAGEN).

Induction of LPS-induced Endotoxin Shock. 10- to 12-wk-old SLPI$^{-/-}$ mice and wild-type littermates (body weight 20–23 g) were intraperitoneally injected with 1.0 mg or 0.5 mg of LPS from *Escherichia coli* serotype 0127:B8 (Sigma-Aldrich). The mice were injected at time 0 and monitored for survival for 5 d. Serum concentrations of IL-6, TNF-α, and IL-1β were measured by ELISA (Endogen).

Induction of Cecal Ligation and Puncture. The CLP was used as a model of systemic sepsis syndrome as described previously (16). Briefly, mice were anesthetized with ketamine hydrochloride (100 mg/kg, intraperitoneally) and xylazine hydrochloride (10 mg/kg, intraperitoneally), and a 1- to 2-cm longitudinal incision was performed to the middle of the abdomen. After the cecum was exposed, its distal one-third point was ligated with a 4–0 silk suture, and its proximal part was punctured twice with a 26-gauge needle. The cecum was then returned into the peritoneal cavity, and the incision was closed by suturing peritoneum and fascia separately with a 4–0 prolene suture to prevent leakage of fluid. The mice were monitored for survival for 5 d.

Preparation of Peritoneal Macrophages and LPS Treatment. Mice were intraperitoneally injected with 2 ml of 4% thioglycollate medium (Sigma-Aldrich). Peritoneal cells were isolated by washing the peritoneal cavity with ice-cold PBS. The cells were cultured for 1 h and nonadherent cells were washed out by PBS. Adherent cells (peritoneal macrophages: 5×10^6) were cultured with LPS (100 ng/ml; Sigma-Aldrich) or LPS and IFN-γ (100 U/ml, PeproTech) in DMEM medium (Sigma-Aldrich) with 10% fetal bovine serum (GIBCO BRL) for 12 or 24 h. Concentrations of IL-6, IL-1β, and TNF-α in the culture supernatants were measured by ELISA (Endogen). Production of NO_2^- was measured by Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical).

B Cell Proliferation Assay. CD19-positive splenic B cells were purified by MACS magnetic cell sorter (Miltenyi Biotec). Purified B cells (3×10^5) were cultured with the indicated concentration of LPS for 24 h. Cells were pulsed with 0.5 Bq of [^{3}H] thymidine (Amersham Biosciences) for the last 8 h. The incorporated radioactivity was measured by a β scintillation counter. Concentration of IgM in the culture supernatants was measured by ELISA (BETHYL INC.).

Western Blot Analysis and Electrophoretic Mobility Shift Assay. The whole cell protein from peritoneal macrophages (5×10^6) was prepared by M-PER Mammalian Protein Extraction Reagent (Pierce Chemical Co.) with protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined by BCA Protein Assay Reagent (Pierce Chemical Co.). Whole cell protein or cell culture medium was denatured by boiling in SDS-PAGE sample buffer, separated on SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was treated with antibodies against mouse SLPI, IκB-α, IκB-β, or HMG-1 (Santa Cruz Biotechnology, Inc.) and the specific bands were detected by BCIP/NBT Phosphatase Substrate System (Kirkegaard & Perry Labs). Electrophoretic mobility shift assay (EMSA) was performed using the Gel Shift Assay System (Promega). Briefly, double-stranded NF-κB (Promega) and C/EBP-β (Santa Cruz Biotechnology, Inc.) consensus oligonucleotides (5 μg each) were end-labeled with [γ−^{32}P]ATP (Amersham Biosciences). Whole cell protein (5 μg) and oligonucleotide were incubated in binding buffer for

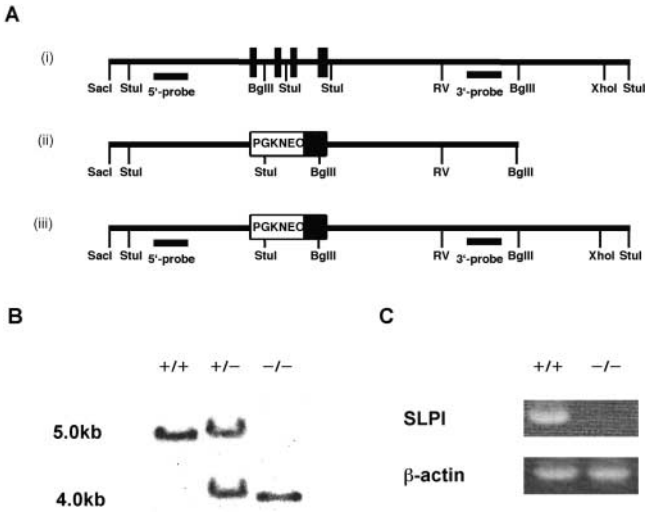

Figure 1. (A-C) Disruption of the SLPI gene by homologous recombination. (A) Structure of the SLPI genomic loci (i), the targeting construct (ii), and the SLPI loci after homologous recombination (iii). (B) Southern blot analysis. Genomic DNA was isolated from mouse liver, digested with StuI and hybridized with a probe located on the 5′ side of the SLPI gene. (C) SLPI expression in the peritoneal macrophages detected by RT-PCR.

20 min. Reaction products were separated in a Novex 6% TBE Gel (Invitrogen) and detected by autoradiography.

Results and Discussion

Generation of SLPI-deficient Mice. To investigate the function of endogenous SLPI in bacterial infection, and thus to clarify the role of SLPI in innate immunity, we have generated a mouse strain lacking SLPI by gene targeting in ES cells. We disrupted the mouse SLPI gene by replacing all four exons with a PGK-NEO expression cassette (Fig. 1 A). The targeting vector was electroporated into ES cells and G418-resistant colonies were isolated. Correctly targeted ES cells were injected into C57BL/6 blastocysts and chimeric mice were generated. The chimeric mice transmitted the mutated allele through the germline, which enables generation of heterozygous mice and homozygous mice by intercrosses. We confirmed the absence of SLPI gene loci by both Southern blot analysis (Fig. 1 B) and RT-PCR (Fig. 1 C). The SLPI$^{-/-}$ mice reproduced normally and appeared healthy without any obvious abnormalities.

Table I. *Survival Rate (%) of the Mice in LPS or CLP-induced Sepsis*

	SLPI$^{+/+}$	SLPI$^{-/-}$
LPS (0.5 mg/mouse)	80% (12/14)	67% (10/15)
LPS (1 mg/mouse)	80% (17/21)	24% (5/21)
CLP	86% (12/14)	43% (6/14)

Mice were injected intraperitoneally with LPS (0.5 mg or 1 mg/mouse) or received CLP. Mice were observed for 5 d.

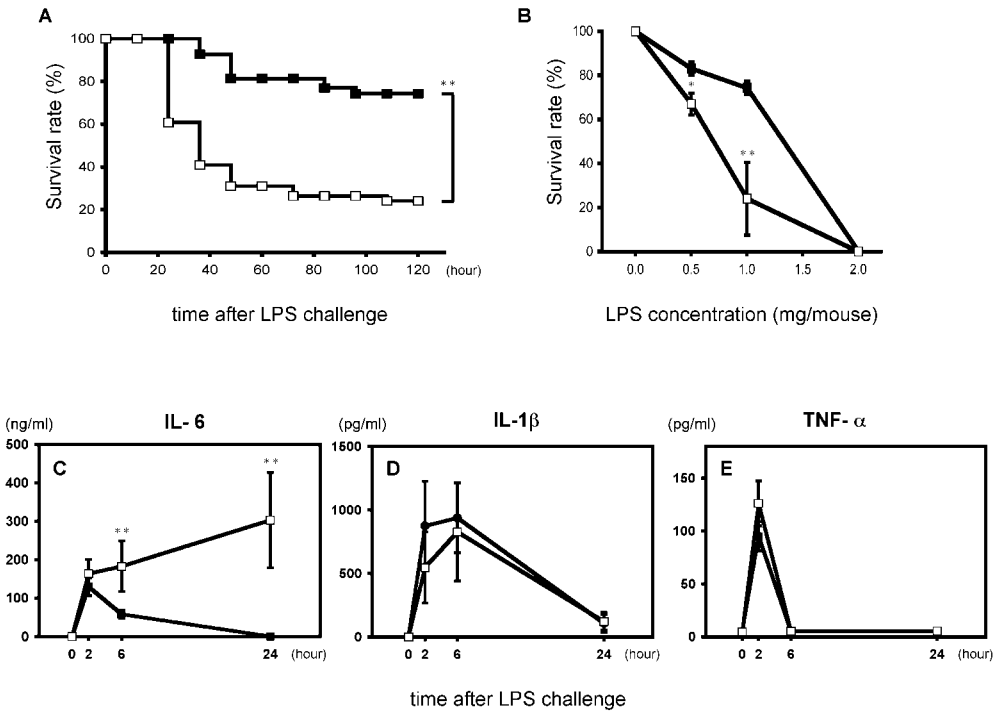

Figure 2. (A) Mortality after LPS administration. Age-matched SLPI$^{+/+}$ mice (■, n = 21) and SLPI$^{-/-}$ mice (□, n = 21) were intraperitoneally injected with LPS (1 mg/mouse). Mortality was assessed every 12 h for 5 d. The experiment was repeated three times and mean values are shown. Statistical analyses were performed using Fisher's exact test: **P < 0.01 between SLPI$^{+/+}$ mice and SLPI$^{-/-}$ mice. (B) Dose–response curves of mortality in SLPI$^{-/-}$ mice (□, n = 10–21) and SLPI$^{+/+}$ mice (■, n = 10–21). The experiment was repeated three times and the results are presented as mean ± SD. Statistical analyses were performed using Fisher's exact test: *P < 0.05, **P < 0.01 between SLPI$^{+/+}$ mice and SLPI$^{-/-}$ mice. (C–E) Serum concentration of cytokines in SLPI$^{-/-}$ mice after LPS injection. SLPI$^{+/+}$ mice (■, n = 6) and SLPI$^{-/-}$ mice (□, n = 6) were intraperitoneally injected with LPS (1 mg/ml). Samples were taken at 2, 6, and 24 h after LPS administration. IL-6 (C), IL-1β (D), and TNF-α (E) concentrations were determined by ELISA. These analyses are representative of three separate experiments. Results are presented as mean ± SD. Statistical analyses were performed using Student's t test: **P < 0.01 between SLPI$^{+/+}$ mice and SLPI$^{-/-}$ mice.

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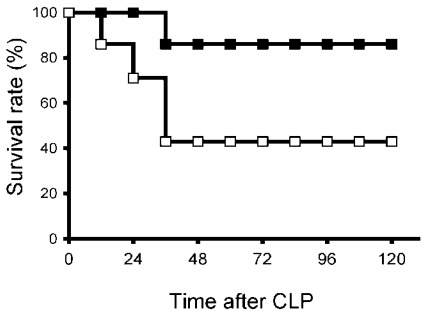

Figure 3. Survival curves after receiving the CLP procedure. Mortality in the age-matched SLPI$^{+/+}$ mice (■, $n = 14$) and SLPI$^{-/-}$ mice (□, $n = 14$) were observed every 12 h for 5 d.

SLPI $^{-/-}$ Mice Are Highly Sensitive to LPS-induced Endotoxin Shock. SLPI$^{-/-}$ mice were subjected to the study of experimental endotoxin shock induced by LPS. SLPI$^{-/-}$ mice and SLPI$^{+/+}$ mice were intraperitoneally injected with LPS (1 mg/mouse), and their survival was then monitored. After the LPS challenge, many of the SLPI$^{+/+}$ mice survived, whereas 76% of the SLPI$^{-/-}$ mice died (Fig. 2 A, Table I). Similar results were obtained in the experiments using lower doses (Fig. 2 B, Table I). Both SLPI$^{-/-}$ and SLPI$^{+/+}$ mice showed a remarkable increase in the serum levels of inflammatory cytokines, IL-6, IL-1β, and TNF-α (Fig. 2, C–E). SLPI$^{-/-}$ mice, however, showed a significantly higher level of IL-6 than wild-type mice at 6 h (Fig. 2 C), suggesting that this may be the cause of the high mortality in SLPI$^{-/-}$ mice after LPS treatment.

Increased Susceptibility to CLP-induced Sepsis in SLPI$^{-/-}$ Mice. SLPI$^{-/-}$ mice were subjected to an in vivo model of sepsis, CLP, in which bacterial peritonitis is induced by surgical perforation of the cecum. As expected, SLPI$^{-/-}$ mice showed a significantly higher lethality than SLPI$^{+/+}$ mice (Fig. 3, Table I). These results conform to the idea that SLPI has a regulatory role against endotoxin shock induced by gram-negative bacteria.

Response to LPS in Macrophages and B Cells of SLPI$^{-/-}$ Mice. The macrophage plays a critical role in the process of endotoxin shock (17). In mammalians, macrophages are the primary responders to LPS and they release numerous bioactive molecules such as inflammatory cytokines, H2O2 and NO. To examine the effect of SLPI deficiency on macrophages, thioglycollate-elicited peritoneal macrophages were stimulated with LPS (100 ng/ml) for 12 or

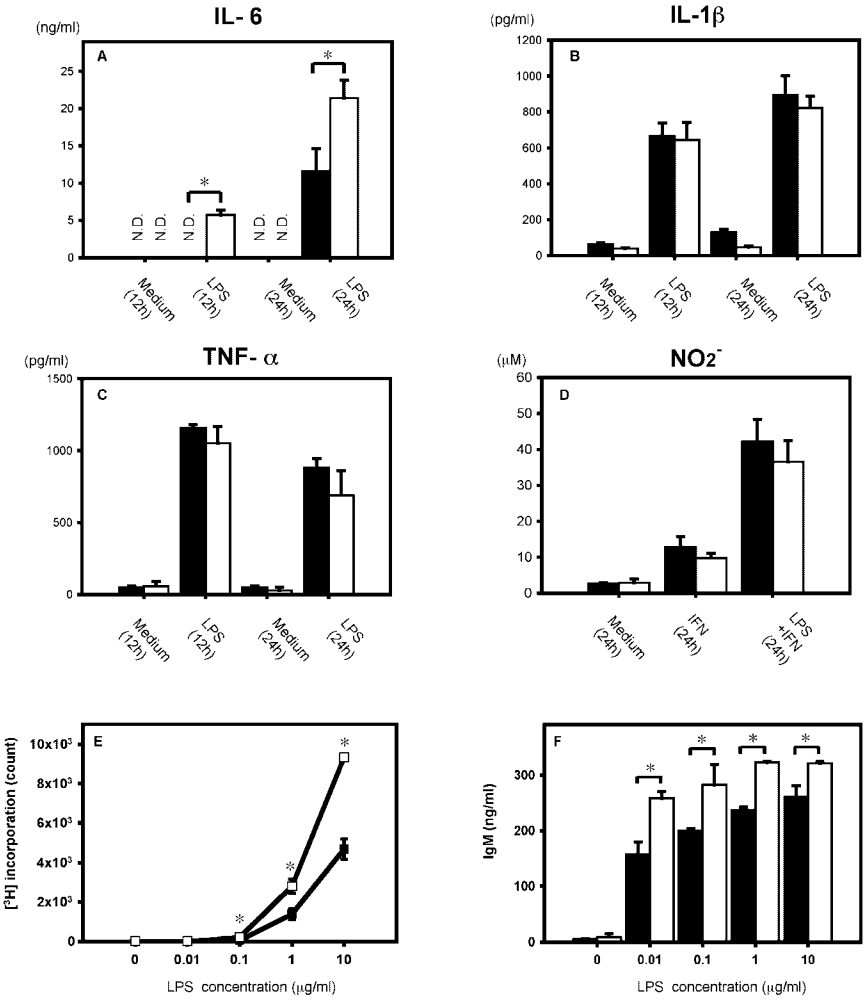

Figure 4. (A-D) LPS-induced production of cytokines and NO2^{-} from SLPI$^{-/-}$ and SLPI$^{+/+}$ macrophages. Thioglycollate-elicited peritoneal macrophages from SLPI$^{-/-}$ mice (□) and SLPI$^{+/+}$ mice (■) were cultured with LPS (100 ng/ml) for 12 or 24 h. Concentrations of IL-6 (A), IL-1β (B), TNF-α (C), and NO_2^- (D) in the supernatants were measured by ELISA. (E and F) Proliferation and IgM production of SLPI$^{-/-}$ B cells in response to LPS. Splenic B cells from SLPI$^{-/-}$ mice (□) and SLPI$^{+/+}$ mice (■) were purified and cultured with the indicated concentrations of LPS for 24 h. (E) Cells were pulsed with 0.5 Bq of [^{3}H] thymidine for the last 8 h. The incorporated radioactivity was measured by a β scintillation counter. (F) Concentrations of IgM in the culture supernatants were measured by ELISA. All analyses are representative of five separate experiments. Results are presented as mean ± SD. N.D., not detected. Statistical analyses were performed using Student's t test: *$P < 0.01$ between SLPI$^{+/+}$ mice and SLPI$^{-/-}$ mice.

24 h, after which the supernatants were harvested and analyzed. Consistent with the in vivo results, macrophages from SLPI$^{-/-}$ mice produced significantly more IL-6 than SLPI$^{+/+}$ mice (Fig. 4, A–D). Moreover, macrophages from SLPI$^{-/-}$ mice showed higher levels of HMG-1, which is a late mediator of endotoxin lethality (18), than SLPI$^{+/+}$ mice (Fig. 5), showing that endogenous SLPI plays a critical role in LPS-induced IL-6 and HMG-1 production.

In response to LPS, B cells secrete SLPI and at the same time begin to proliferate and to produce immunoglobulins. To evaluate the SLPI action on the activation of B cells, splenic B cells were cultured in the presence of different concentrations of LPS. B cells from mice lacking SLPI showed a stronger proliferative response to LPS than did B cells from their wild-type littermates (Fig. 4 E). In addition, B cells from SLPI$^{-/-}$ mice produced more IgM than those from SLPI$^{+/+}$ mice (Fig. 4 F). These results indicate that SLPI inhibits the activation of B cells. On the other hand, the proliferative response of B cells upon B cell receptor (BCR) stimulation showed no differences between SLPI$^{-/-}$ and SLPI$^{+/+}$ mice (data not shown).

Inhibitory Effects on LPS Signaling Pathways by SLPI. Recently, TLR4 was identified as a macrophage receptor for LPS (19). After associating with CD14, TLR4 recognizes LPS and binds to it. TLR4 then activates various signaling molecules involving MyD88, IL-1R–associated kinase (IRAK), TRAF6, and Iκkinase (IκK), leading to the degradation of IκB. After the degradation of IκB, NF-κB enters the nucleus and then exerts its action (1, 2). This pathway is regulated at multiple points, e.g., TLR4 on the cell surface of macrophage is down-regulated after LPS treatment (20). Data showing that recombinant SLPI directly interferes with the LPS–CD14 complex formation (21) and that overexpression of SLPI in macrophages suppresses LPS-induced NF-κB activation (22) provide evidence that SLPI affects this pathway. The inhibitory effects of SLPI on LPS signaling, however, are still controversial because administration of recombinant SLPI has no effect on macrophage responsiveness to LPS (22). To investigate the role of SLPI on the signaling pathway, we first examined the surface expression of TLR4 and CD14. Macrophages from SLPI$^{-/-}$ mice (SLPI$^{-/-}$ macrophages) and from wild-type mice (SLPI$^{+/+}$ macrophages) were cultured with LPS for 24 h and analyzed by flow cytometry. Contrary to the previous report (21), SLPI$^{-/-}$ macrophages had no effect on CD14 or TLR4 expression (data not shown).

In the signaling pathway, SLPI$^{-/-}$ macrophages and wild-type macrophages showed similar levels of IκB-α expression during LPS treatment (Fig. 6 A). IκB-β expression in SLPI$^{-/-}$macrophages remained suppressed at both 30 and 60 min, while that in SLPI$^{+/+}$ macrophages was restored at 60 min (Fig. 6 A). DNA binding activity of NF-κB in SLPI$^{-/-}$ macrophages was stronger than that in SLPI$^{+/+}$ macrophages during LPS stimulation, especially at 60 and 120 min (Fig. 6 B). Moreover, C/EBP-β activation of SLPI$^{-/-}$ macrophages was also higher than that of SLPI$^{+/+}$ macrophages at 120 min (Fig. 6 B). These results indicate that SLPI suppresses the activation of NF-κB as well as C/EBP-β, which is, at least in part, mediated by the regulation of the IκB-β level, and also suggest that the increased IL-6 production in SLPI$^{-/-}$ mice depends upon

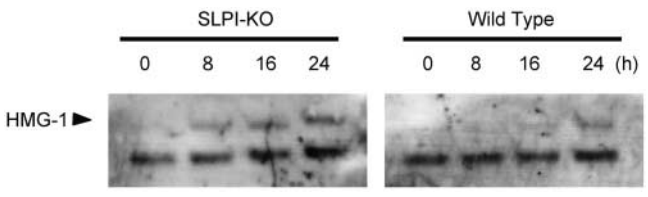

Figure 5. LPS-induced production of HMG-1 from SLPI$^{-/-}$ and SLPI$^{+/+}$ macrophages. Peritoneal macrophages from SLPI$^{-/-}$ and SLPI$^{+/+}$ mice were stimulated with LPS (100 ng/ml) for the indicated periods. HMG-1 protein in the culture medium was detected by Western blot analysis. The results are representative of three separate experiments.

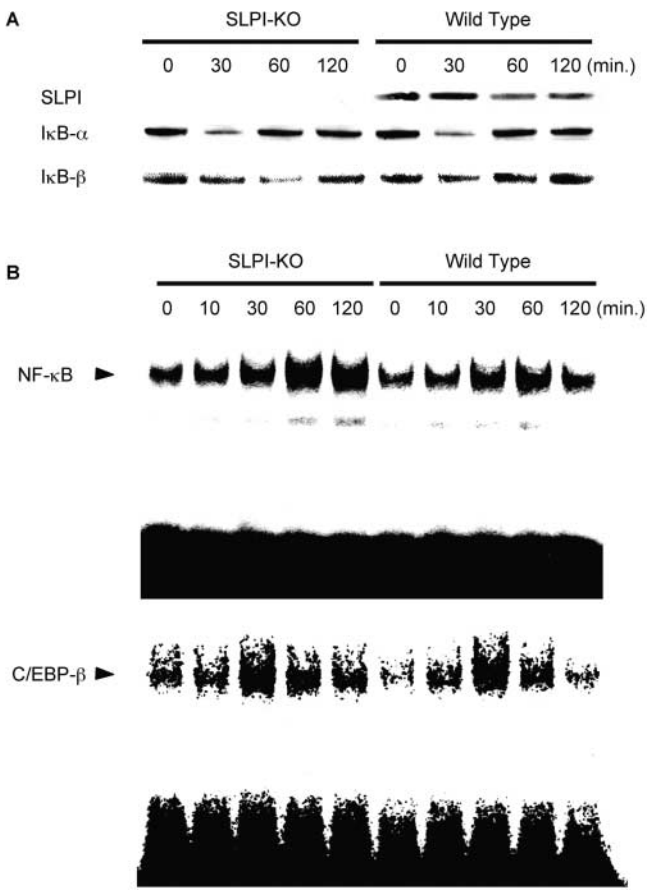

Figure 6. (A) Changes in the expression of mouse SLPI, IκB-α, and β in response to LPS in macrophages from SLPI$^{-/-}$ and SLPI$^{+/+}$ mice. Peritoneal macrophages from SLPI$^{-/-}$ and SLPI$^{+/+}$ mice were stimulated with LPS (100 ng/ml) for the indicated periods. Western blot analysis was performed for SLPI (top panel), IκB-α (middle) and β (bottom). (B) NF-κB and C/EBP-β activities in response to LPS in macrophages from SLPI$^{-/-}$ and SLPI$^{+/+}$ mice. The whole cell lysate (5 μg protein) from either SLPI$^{-/-}$ or SLPI$^{+/+}$ was incubated with a specific NF-κB or C/EBP-β probe, and NF-κB (top) and C/EBP-β (bottom) activity was determined by the electrophoretic mobility shift assay (EMSA). The results are representative of three separate experiments.

the elevation of the activities of both NF-κB and C/EBP-β. In support of our observation, administration of recombinant SLPI inhibited LPS-induced IκB-α and β degradation (23).

In conclusion, our present study for the first time provides evidence that SLPI$^{-/-}$ mice are highly sensitive to LPS or CLP, and demonstrates that SLPI inhibits the LPS signaling pathway through suppression of NF-κB and activation of C/EBP-β. SLPI counteracts excessive inflammatory responses and thus assures the adequate functioning of innate immunity.

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