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Secretory Leukocyte Protease Inhibitor: A Macrophage Product Induced by and Antagonistic to Bacterial Lipopolysaccharide

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Summary

To explore regulation of potentially lethal responses to bacterial lipopolysaccharide (LPS), we used differential display under LPS-free conditions to compare macrophage cell lines from two strains of mice congenic for a locus affecting LPS sensitivity. LPS-hyporesponsive cells, primary macrophages, and polymorphonuclear leukocytes transcribed secretory leukocyte protease inhibitor (SLPI), a known epithelial cell-derived inhibitor of leukocyte serine proteases. Transfection of macrophages with SLPI suppressed LPS-induced activation of NF-κB and production of nitric oxide and TNFα. The ability of interferon-γ (IFNγ) to restore LPS responsiveness is a hallmark of the LPS-hyporesponsive phenotype. IFNγ suppressed expression of SLPI and restored LPS responsiveness to SLPI-producing cells. Thus, SLPI is an LPS-induced IFNγ-suppressible phagocyte product that serves to inhibit LPS responses.

Introduction

Endotoxic lipopolysaccharide (LPS), the major constituent of the cell walls of Gram-negative bacteria, is among the most potent substances in biology. A few molecules per cell can trigger macrophages, the most LPS-responsive cells in the mammalian host, to release a battery of alarm signals and defense molecules, including TNFα, IL-1, IL-12, migration inhibitory factor (MIF), chemokines, interferons, eicosanoids, and reactive oxygen and nitrogen intermediates (Ding and Nathan, 1987; Nathan, 1987). The macrophage secretory response to LPS can protect the host from infection, but can also cause tissue injury, cachexia, circulatory collapse, multiple organ failure, and death (Parker and Parrillo, 1983; Bone, 1991). Thus, both biological and medical questions motivate inquiry into the regulation of responses to LPS.

More is known about stimulation of LPS responses than about their inhibition. LPS binding protein (Tobias et al., 1986) catalyzes the formation of complexes of LPS with the glycophosphatidyl inositol-linked receptor, CD14 (Wright et al., 1990). These complexes interact with an unidentified coreceptor (Ulevitch and Tobias, 1995) to activate tyrosine kinases, proline-directed serine/threonine kinases (such as ERK1, ERK2, p38α, SAPK, and ceramide-activated kinase), other serine/threonine kinases (such as Raf, protein kinase C, and protein kinase A), GTPases (such as ras), and transcription factors (such as NF-κB) (Sweet and Hume, 1996). Cytokines can enhance (IFNγ) or suppress (TGFβ, IL-10, IL-4, IL-11, IL-13) the macrophage's response to LPS.

In mice, responsiveness to LPS is controlled by an unidentified gene located on chromosome 4. A defect in this locus in C3H/HeJ mice confers LPS resistance (Sultzer, 1968; Watson et al., 1978; Sultzer et al., 1993). Compared to an ostensibly congenic strain, C3H/HeN, which carries normal alleles (Lps^n) of the *Lps* gene, C3H/HeJ (Lps^d) mice are hyporesponsive to LPS, a phenotype expressed in macrophages, B lymphocytes, T lymphocytes, and fibroblasts (Rosenstreich and Glode, 1975; Sultzer, 1976; Beutler et al., 1986a). Moreover, C3H/HeJ mice survive injection of LPS at doses 20–40 times higher than those lethal to C3H/HeN mice (Sultzer, 1968). The LPS defect in C3H/HeJ mice is not absolute, since certain partial responses are preserved under the usual conditions of stimulation (Coutinho et al., 1975), while others are evoked by higher concentrations of, or after longer exposures to, LPS (Sultzer, 1968; Ding et al., 1995). Strikingly, coadministration of interferon-γ (IFNγ) normalizes the LPS responsiveness of C3H/HeJ mice (Beutler et al., 1986b). The mechanism of compensation by IFNγ is as obscure as the biochemical basis of the defect itself.

In an effort to learn more about regulation of responsiveness to LPS, we carried out differential display analysis (Liang and Pardee, 1992) on matched macrophage cell lines from C3H/HeJ and C3H/HeN mice. We reasoned that differential display should be unbiased with respect to whether the defect in the hyporesponsive strain represents extinction of an LPS response mediator or overexpression of an LPS response inhibitor. We compared cultured cell lines as one of the only secure routes to studying genes whose expression differs in the absence of LPS, a stimulus to which most primary macrophages in conventionally reared mice can be presumed to have been exposed. Described below is the cloning of cDNA for *secretory leukocyte protease inhibitor* (*SLPI*), a gene overexpressed in a C3H/HeJ (Lps^d) macrophage cell line but inducible by LPS in wild-type macrophages and neutrophils, and suppressible by IFNγ. The *SLPI* gene product is a protein that was previously well studied to the point of structural solution, and yet about which it was not known that macrophages produce it, that its production is regulated by LPS and IFNγ, and that its actions include antagonism of LPS-induced signaling and secretion.

Results

SLPI Is Constitutively Overexpressed in a Macrophage Cell Line from C3H/HeJ (Lps^d) but Not C3H/HeN (Lps^n) Mice

We compared gene expression patterns in macrophage cell lines, cultured under LPS-free conditions, which

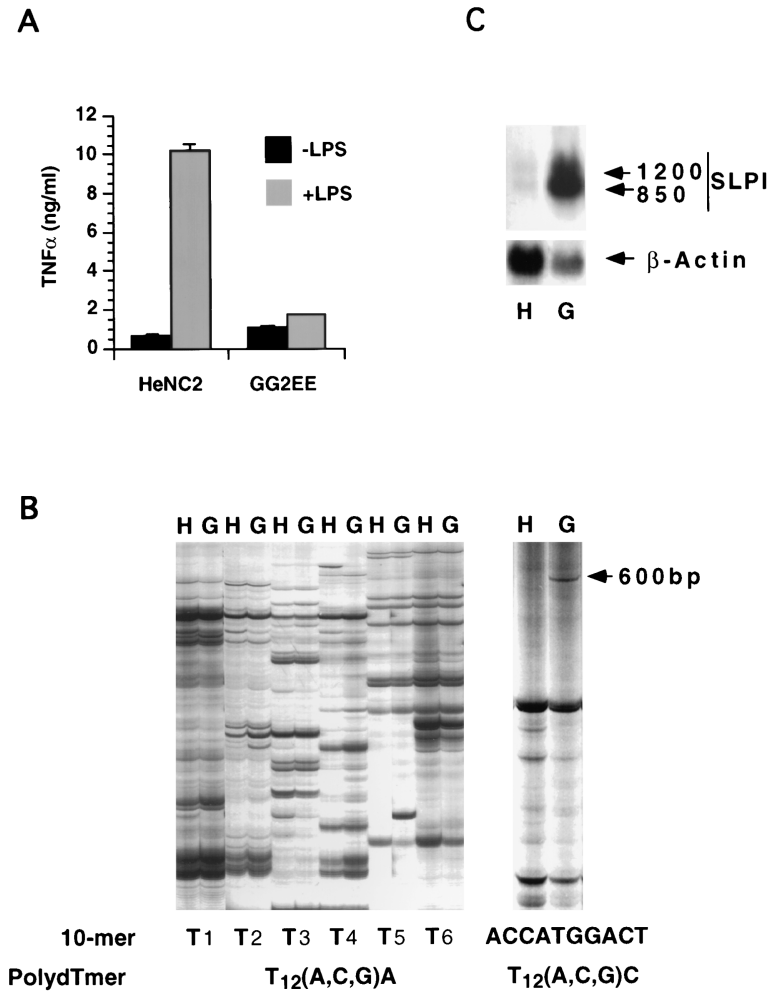

Figure 1. Identification of a Gene (SLPI) Overexpressed in LPS-Hyporesponsive GG2EE (*Lps*^{d}) Cells Compared to LPS-Normoresponsive HeNC2 (*Lps*^{n}) Cells

(A) Differential responsiveness of HeNC2 (*Lps*^{n}) and GG2EE (*Lps*^{d}) cells to LPS. Cells (10^5 per well) were treated for 24 hr with or without 1 ng/ml of LPS and TNFα in the supernatants determined by ELISA. Results are means ± SEM of triplicates from one of four similar experiments.

(B) Examples of differential display (left panel) using as one primer either T1 (AGCCAGC GAA), T2 (CAAAGGGAGA), T3 (GAAGTGG TTT), T4 (CAGTCAACCT), T5 (CTCAACCTCC), or T6 (CTGATCCATG), and T_{12}MA as the other. In each pair of lanes, the left (H) is amplified from HeNC2 (*Lps*^{n}) cells and the right (G) from GG2EE (*Lps*^{d}) cells. In the right panel, SLPI (marked by arrow) was amplified only from GG2EE (*Lps*^{d}) cells using the primers ACCATGGACT and T_{12}MC.

(C) Northern blot with total RNA (20 μg) from HeNC2 (*Lps*^{n}) and GG2EE (*Lps*^{d}) cells probed with mouse SLPI cDNA fragment isolated by differential display, confirming its differential expression. The same membrane was rehybridized with a β-actin oligonucleotide probe as a loading control.

were derived from two mouse strains believed to be *Lps* congenics: C3H/HeN (*Lps*^{n}) (HeNC2 cells) and C3H/HeJ (*Lps*^{d}) (GG2EE cells). These cell lines maintain the LPS-responsive (HeNC2) or LPS-hyporesponsive (GG2EE) phenotypes of their parental cells with respect to LPS-induced cytotoxicity toward L5178Y target cells and LPS-induced TNFα release (Blasi et al., 1987) (Figure 1A). Each primer set for differential display consisted of a random 10-mer and a T_{12}M(C, G, T, A), where M stands for the mixture of C, G, A. About 50–100 amplification products from one PCR reaction were distinguishable on a 5% polyacrylamide gel. As illustrated for seven primer sets in Figure 1B, more than 99.75% of 12,000 such products were shared by the HeNC2 (*Lps*^{n}) and GG2EE (*Lps*^{d}) cells. Only four mRNAs (two in each of the cell lines) were consistently expressed in a differential manner in multiple replicate experiments. The present study concerns a transcript amplified with the primer set ACCATGGACT and T_{12}MC that was expressed only by GG2EE (*Lps*^{d}) cells and not by HeNC2 (*Lps*^{n}) cells. This amplification product was isolated, reamplified, cloned, and sequenced. The primer sequences flanked a previously unreported reading frame that was 68% homologous to human *SLPI*. Northern blot analysis with the mouse *SLPI* probe confirmed a higher level of expression in GG2EE (*Lps*^{d}) cells than in HeNC2 (*Lps*^{n}) cells (Figure 1C).

Cloning of Mouse *SLPI*

The subcloned cDNA fragment of mouse *SLPI* was used as probe to screen a cDNA library from IFNγ- and LPS-activated RAW 264.7 cells, an Abelson–virus transformed murine macrophage cell line. This previously prepared library (Xie et al., 1992) was used even though RAW 264.7 cells respond normally to LPS and express much less *SLPI* than GG2EE cells, as documented below. The full-length 1123 bp mouse *SLPI* cDNA was comprised of a 5′ untranslated region (nucleotides 1–446), open reading frame (447–842), and 3′ untranslated region (843–1123). Overall homology with human SLPI (Thompson and Ohlsson, 1986) is 68% at the nucleotide level and 60% at the amino acid level (Figure 2). The signal peptides are particularly well conserved (80%). In contrast, the secreted proteins are only 52% identical. However, all 16 cysteine residues are preserved.

Cell Lines That Overexpress SLPI Are Hyporesponsive to LPS

Since GG2EE (*Lps*^{d}) cells express SLPI and resist LPS, while HeNC2 (*Lps*^{n}) cells express almost no SLPI and

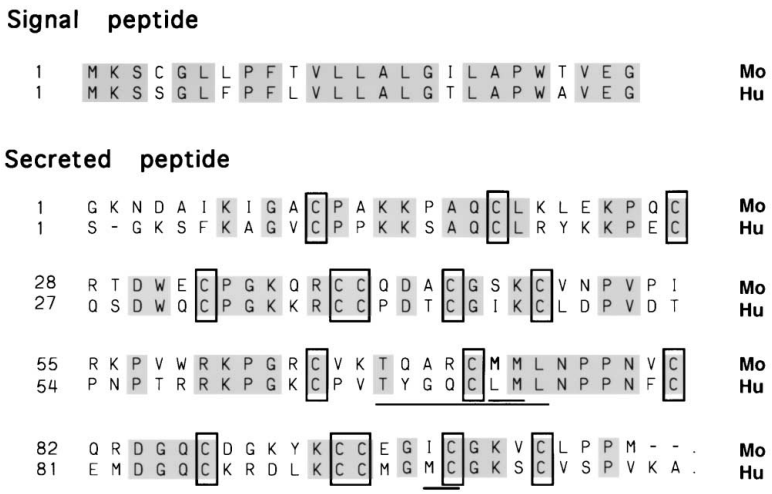

Figure 2. Amino Acid Sequence Comparison between Mouse and Human SLPI

Translated amino acid sequence of mouse (Mo) SLPI is aligned with human (Hu) SLPI. Conserved residues are shaded; Cys are boxed. The binding region for proteases is underlined and the reactive site is double-underlined with boldface marking the substitution of Leu (human) with Met (mouse).

respond to LPS, we compared levels of SLPI transcript and protein with sensitivity to LPS in three additional macrophage cell lines from other genetic backgrounds (ANA-1, C57BL/6; RAW 264.7 and J774.1, BALB/c) (Figure 3). Secreted SLPI was detected by immunoprecipitation with antibody raised against recombinant mouse SLPI. RAW 264.7 and ANA-1 cells expressed as little *SLPI* mRNA and secreted as little SLPI protein as HeNC2 (*Lps*^{n}) cells. As shown earlier, GG2EE (*Lps*^{d}) cells expressed a high level of *SLPI* mRNA; in addition, they

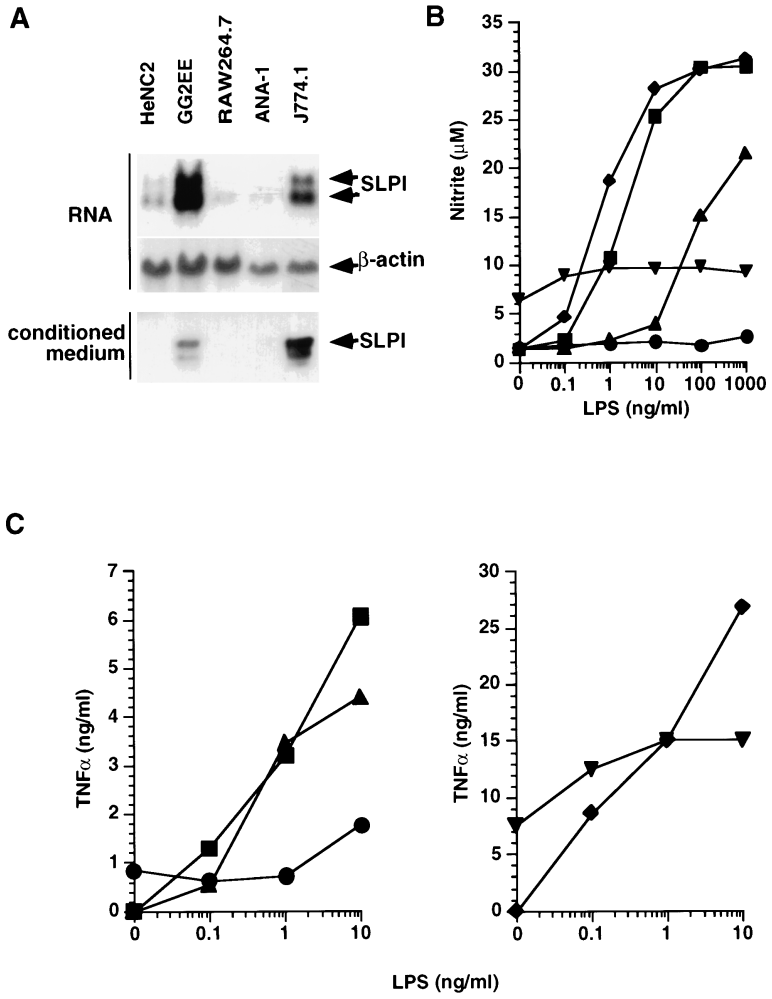

Figure 3. Relationship between Expression of SLPI and Production of NO and TNFα in Response to LPS in HeNC2 (*Lps*^{n}), GG2EE (*Lps*^{d}), RAW 264.7, ANA-1, and J774.1 Cells

(A) Upper: Northern blot (20 μg RNA) probed with SLPI cDNA. The same membrane was reprobed with a β-actin oligonucleotide as a loading control. Lower: Immunoblot of SLPI in the medium of cultures of the indicated cells after immunoprecipitation with rabbit-anti-mouse-SLPI serum.

(B) NO production in response to LPS. HeNC2(■), GG2EE(●), ANA-1(▲), RAW264.7 (◆), and J774.1(▼) cells (10^5 per well) were exposed to LPS for 48 hr. Nitrite accumulation from the conditioned media was determined. Results are means of triplicates. Error bars indicating SEM fall within the symbols. One of five similar experiments is shown.

(C) TNFα production in response to LPS. Cells as in (B) were exposed to LPS for 12 hr. TNF content in the supernatants was determined by ELISA. Results are from one of three similar experiments.

secreted abundant SLPI protein. J774.1 expressed an intermediate level of *SLPI* mRNA, but secreted the most protein of the five macrophage lines tested (Figure 3A).

Reciprocally, HeNC2, ANA-1, and RAW 264.7 cells (all *Lps*^{n}) responded to as little as 10 ng/ml LPS by releasing 20- to 40-fold more NO and TNFα than they secreted spontaneously. By the same criteria, GG2EE (*Lps*^{d}) and J774.1 cells were LPS-resistant (Figures 3B and 3C). Although J774.1 cells released some NO and TNF in the absence of LPS, the amount scarcely changed with increasing concentrations of LPS.

Transfection with SLPI Converts Macrophages from LPS-Sensitive to LPS-Hyporesponsive

The inverse correlation between SLPI expression and LPS hyporesponsiveness among five macrophage cell lines prompted us to test whether expression of SLPI causes LPS hyporesponsiveness. HeNC2 (*Lps*^{n}) cells were transfected with the p463-neo control vector or the same vector containing the *SLPI* open reading frame (p436-neo-SLPI). Stably transfected clones were phenotyped by Northern and Western analyses. HeNC2-C2C7 (transfected with p463-neo vector only) expressed no SLPI, while the independently selected clones HeNC2-C6C10 and HeNC2-D4F9 (both transfected with p436-neo-SLPI) expressed equivalent levels of *SLPI* mRNA and protein as GG2EE (*Lps*^{d}) cells (Figure 4A). LPS responsiveness, judged by induction of secretion of NO and TNFα, was preserved in HeNC2-C2C7 cells (control vector-transfected), just as in the parental HeNC2 (*Lps*^{n}) cells. In contrast, the two SLPI transfectants became markedly LPS hyporesponsive, requiring 100-fold more LPS to produce the same amounts of the LPS-response products as the parental cells (Figure 4B).

To evaluate signaling by LPS, we performed electrophoretic mobility shift assays with an NF–κB binding element shown to be essential for LPS to induce transcription of the high-output form of nitric oxide synthase (iNOS; NOS2) (Xie et al., 1994). As little as 100 pg/ml LPS activated NF-κB in parental HeNC2 (*Lps*^{n}) cells and in mock-transfected cells, but LPS failed to do so in GG2EE (*Lps*^{d}) cells or in the two SLPI transfectants of HeNC2 (Figure 4C). The inhibitory effect of SLPI on LPS signaling appears to be specific, since overexpression of SLPI had no effect on IFNγ-induced DNA-binding activity to a gamma response region (GRR) present in the promoter of the Fcγ receptor (Pearse et al., 1991) (Figure 4C).

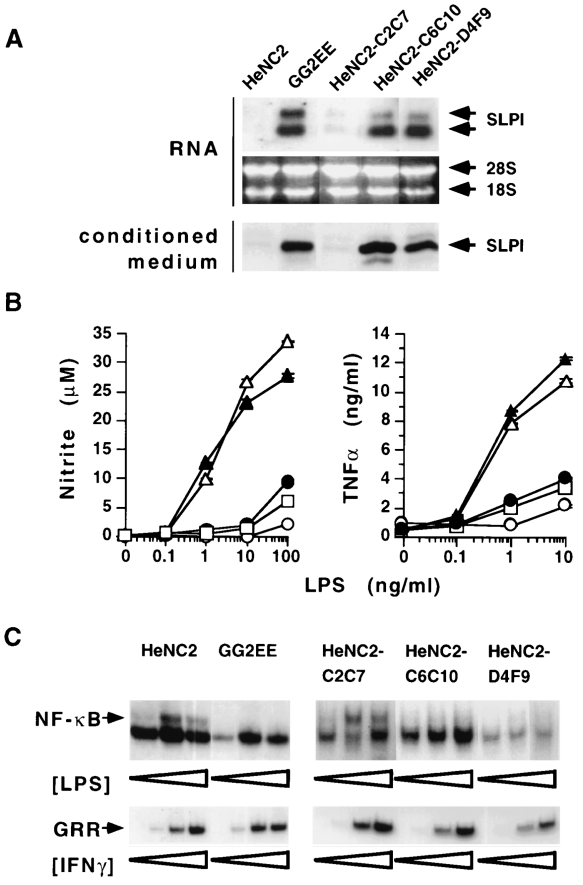

Figure 4. Inhibition of LPS- but not IFNγ-Signaling in SLPI-Overexpressing Clones

(A) Upper: Expression of SLPI mRNA by stable clones of HeNC2 cells (*Lps*^{n}) transfected with p463-neo (HeNC2-C2C7) or with p463-neo-SLPI (HeNC2-C6C10 and HeNC2-D4F9) in comparison to parental HeNC2 cells (*Lps*^{n}) and GG2EE cells (*Lps*^{d}). Northern blot with SLPI cDNA as a probe. Total RNA (20 μg) was loaded in each lane with ethidium bromide staining to control for loading. Lower: Secretion of SLPI. SLPI was immunoprecipitated with rabbit-anti-SLPI antibody from culture medium and immunoblotted with the same antibody.

(B) LPS-responsiveness of HeNC2(△), GG2EE(○), HeNC2-C2C7(▲), HeNC2-C6C10(●), and HeNC2-D4F9(□) in (A) judged by LPS-induced release of nitrite (48 hr) or TNFα (12 hr) as in the legend to Figure 3B. Results are means ± SEM from one of five similar experiments.

(C) Cells were treated with 0, 0.1, or 1 ng/ml of LPS (upper) or 0, 0.1, 1, or 10 U/ml of IFNγ (lower) for 30 min. NF-κB (upper) or GRR (lower) activity in nuclear extracts was determined by EMSA. Arrows indicate position of NF-κB heterodimers and GRR binding complex.

Restoration of LPS Responsiveness in SLPI-High Expressor Cells upon Treatment with IFNγ

The ability of IFNγ to restore LPS responsiveness is a hallmark of the defect in C3H/HeJ mice (Beutler et al., 1986b). As shown in Figure 5A, IFNγ restored LPS-induced NO and TNFα production in GG2EE (*Lps*^{d}) cells. Likewise, IFNγ shifted the LPS concentration–response curve for NO production to the left by two to three orders of magnitude in each of the two independently derived SLPI transfectants (Figure 5B), as well as for TNFα production (data not shown).

Expression of SLPI in Primary Macrophages, Polymorphonuclear Leukocytes, and Organs

The work to this point was centered on cultured cell lines for two reasons: scrupulously to avoid exposure to LPS prior to its addition as a reagent at defined concentrations, and to permit transfection of candidate regulatory genes into the cells under study. Next, we turned to primary cells from C3H/HeN (*Lps*^{n}) and C3H/HeJ (*Lps*^{d}) mice. *SLPI* mRNA was detected in peritoneal macrophages from both strains of mice elicited by intraperitoneal injection of thioglycollate broth (Figure 6A). As is

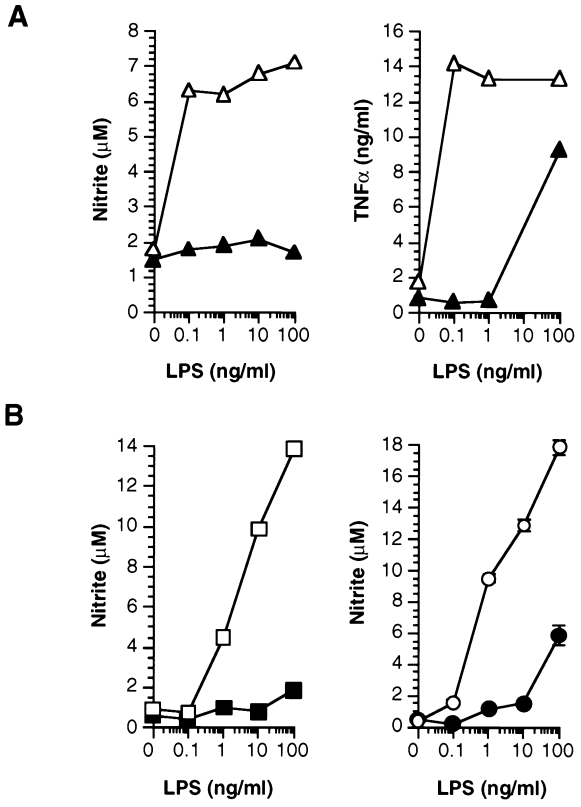

Figure 5. Restoration of LPS Responses in SLPI-Expressing Cells by IFNγ

(A) GG2EE cells (*Lps*^{d}) were incubated in the presence (△) or absence (▲) of IFNγ (10 units/ml) along with the indicated concentrations of LPS and accumulation of nitrite (left panel) or TNFα (right panel) measured at 24 hr.

(B) Two SLPI-expressing transfectants, HeNC2-C6C10 (●,○) and HeNC2-D4F9 (■,□), were treated with (○,□) or without (●,■) IFNγ as in (A), and nitrite accumulation was measured. In all four panels results are means of triplicates from one of four similar experiments. Most of the bars indicating SEM fall within the symbols.

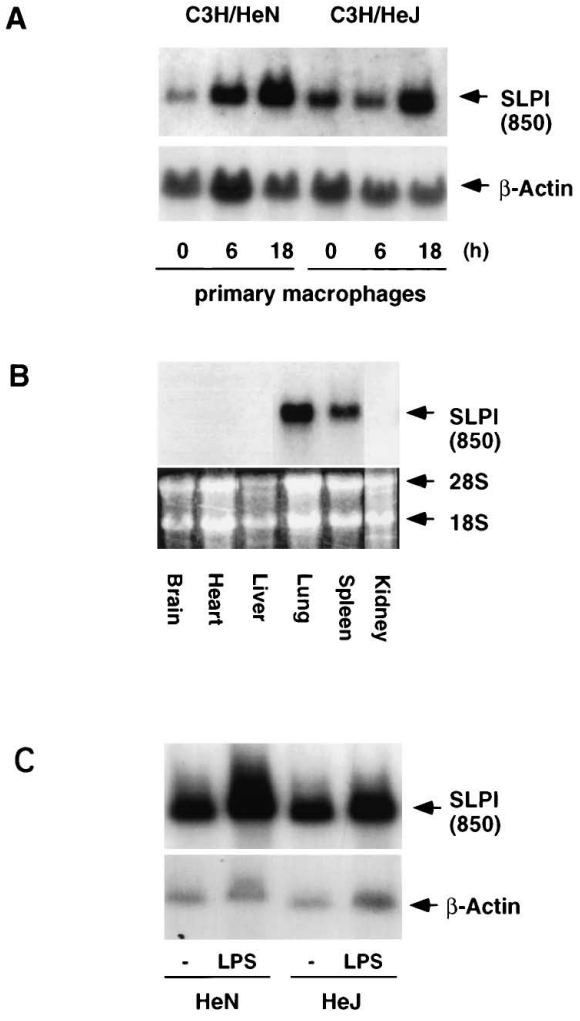

Figure 6. Modulation of SLPI Expression by LPS in Primary Macrophages and PMN

(A) Peritoneal macrophages were collected from from C3H/HeN (*Lps*^{n}) or C3H/HeJ (*Lps*^{d}) mice four days after injection of LPS-contaminated thioglycollate broth and not further treated (0 hr) or cultured with 100 ng/ml of LPS for the indicated times before total RNA was analyzed by northern blot using *SLPI* cDNA as probe (48 hr exposure). The same membrane was reblotted using β-actin oligonucleotide probe as a control.

(B) Expression of *SLPI* transcripts in 20 μg of total RNA from organs of C3H/HeN (*Lps*^{n}) mice four days after intraperitoneal injection of thioglycollate broth with *SLPI* cDNA as probe.

(C) Expression of SLPI in PMN. PMN were collected from peritoneal cavity of C3H/HeN (*Lps*^{n}) mice at 12 hr following injection of thioglycollate broth. PMN were treated with 0 or 100 ng/ml LPS for 4 hr. Six micrograms total RNA was probed with mouse *SLPI* cDNA.

often the case, the thioglycollate broth used to elicit these macrophages was contaminated with LPS (~0.5 ng/ml). In vitro, reagent LPS increased the expression of *SLPI* mRNA in C3H/HeN (*Lps*^{n}) macrophages at 6 and 18 hr. Although no increase in *SLPI* mRNA expression was detected in C3H/HeJ (*Lps*^{d}) macrophages at 6 hr, it became apparent by 18 hr (Figure 6A).

Next, we looked at organs of C3H/HeN (*Lps*^{n}) mice that had been injected intraperitoneally with thioglycollate broth and thus with LPS. Mouse *SLPI* mRNA was expressed not only in lung, as previously noted for human *SLPI* (De Water et al., 1986; Sallenave et al., 1993), but also in spleen (Figure 6B). No *SLPI* transcripts were detected in liver, brain, kidney, or heart. When splenocytes were isolated, the nonadherent, lymphocyte-rich fraction showed little *SLPI* expression. Neither concanavalin A, a T cell mitogen, nor LPS, a B cell mitogen, induced SLPI in lymphocytes (data not shown). In contrast, the other major class of phagocytes, i.e., polymorphonuclear leukocytes (PMN), expressed *SLPI* mRNA after inflammation of the peritoneal cavity by injection of LPS-contaminated thioglycollate broth, and their expression of SLPI increased further upon additional treatment for 4 hr with LPS in vitro, but not with formylated peptide (Figure 6C).

SLPI mRNA was detected in tissue, primary macrophages, PMN, and the ANA-1 and RAW 264.7 cell lines as a single species of 850 nt (Figures 3A, 6, and 7), whereas it appeared as a doublet of 850 and 1200 nt in HeNC2 (*Lps*^{n}), GG2EE (*Lps*^{d}), and J774.1 cells (Figures

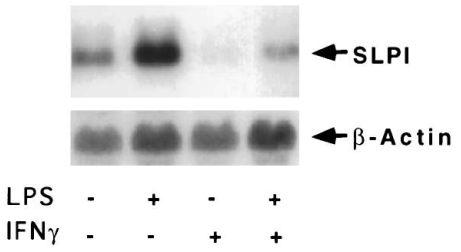

Figure 7. Suppression of SLPI Expression by IFNγ

Northern blot with total RNA (20 μg per lane) from RAW264.7 cells that had been treated in the presence or absence of LPS (100 ng/ml) or IFNγ (10 U/ml) for 4 hr was probed with mouse SLPI cDNA. The same membrane was reblotted using β-actin oligonucleotide probe as a control.

3A and 4A). Whether the additional band represents a splicing variant has not yet been established.

Suppression of *SLPI* Expression by IFNγ

Many differentiative effects of LPS on macrophages are mimicked or augmented by cotreatment with IFNγ. However, because IFNγ augmented LPS responses in *SLPI*-expressing cells, and because SLPI inhibits LPS responses, it was necessary to consider whether IFNγ might decrease the expression of *SLPI*, even though LPS itself had the opposite effect. As shown in Figure 7, exposure of RAW 264.7 cells to LPS led to increased levels of *SLPI* mRNA, while treatment with IFNγ inhibited both the basal and LPS-induced expression of *SLPI*.

Discussion

Mouse SLPI can now be considered a phagocyte-derived LPS-induced LPS inhibitor. New sources, functions, and modes of regulation are surprising additions to the understanding of a protein whose human homolog has been so intensively studied.

SLPI: Sources and Structures

Human SLPI, cloned a decade ago at the cDNA and genomic levels (Heinzel et al., 1986; Seemuller et al., 1986; Stetler et al., 1986), is an 11.7 kDa protein originally isolated from parotid saliva (Thompson and Ohlsson, 1986). Human SLPI was subsequently found in seminal plasma (Ohlsson et al., 1995) and cervical (Helmig et al., 1995), nasal, and bronchial (Hutchison, 1987; Lee et al., 1993) mucus. SLPI was named for its location in secretions and its actions as a potent inhibitor of leukocyte serine proteases, notably elastase and cathepsin G from neutrophils, and chymase and tryptase from mast cells, as well as trypsin and chymotrypsin from pancreatic acinar cells (Fink et al., 1986; Thompson and Ohlsson, 1986; Ohlsson et al., 1988).

Thus, the term "leukocyte" in SLPI's name refers to the source of some of the proteases it inhibits; SLPI itself has been considered exclusively an epithelial cell product (Abe et al., 1991), except for one report of its expression in human neutrophils (Bohm et al., 1992). In mouse, we found SLPI transcript and protein in splenic adherent cells, peritoneal macrophages, bone marrow-derived macrophage cell lines, and peritoneal PMN. Whether human phagocytes likewise express SLPI is being reexamined.

Though the overall amino acid identity of mature mouse and human SLPI is only 52%, there are striking structural similarities. X-ray diffraction analysis of human SLPI revealed a boomerang-shaped molecule comprised of two topologically superimposable domains (Ser^{1}–Pro^{54} and Asn^{55}–Ala^{107}) (Grutter et al., 1988). Mouse SLPI can likewise be envisioned as comprised of two domains (Gly^{1}–Arg^{55} and Lys^{56}–Met^{106}), each of which preserves the spacing of eight cysteine residues characteristic of human SLPI (Seemuller et al., 1986). In human SLPI, all 16 cysteines are engaged in interdomain disulfide links (Grutter et al., 1988). A four disulfide core is thought to stabilize a molecule that lacks a hydrophobic core and has little hydrogen-bonded secondary structure (Drenth et al., 1980). Another hallmark of human SLPI is the abundance of proline (12 per 107 residues), with Pro^{76}-Pro^{77} adopting a polyproline II-like conformation. Of the 11 prolines in mouse SLPI, 9 reside at corresponding positions, including those homologous to Pro^{76}-Pro^{77}. Thus, it is likely that secreted mouse SLPI adopts the same structure as its human counterpart.

However, there may be important differences. X-ray crystallography (Grutter et al., 1988) and mutagenesis analysis (Eisenberg et al., 1990) identified Leu^{72}-Met^{73} in human SLPI as the reactive site for inhibition of elastase, trypsin, and chymotrypsin. In mouse SLPI, the corresponding residues are Met^{73}-Met^{74}. In addition, human SLPI binds some target proteases through the residues TYGQCLML at positions 67–74 plus Met-Cys at 96–97. Mouse SLPI replaces these contacts with TQARCMML (amino acids 68–75), and substitutes Ile for Met at residue 96. We have been unable to express recombinant SLPI in bioactive form, probably because the critical sulfhydryls are susceptible to oxidation and incorrect disulfide bonding (Stolk et al., 1993; Tomova et al., 1994). Thus, we have not yet been able to test if the differences in primary sequence abolish the antiprotease function of mouse SLPI, or, conversely, adapt mouse SLPI to mouse proteases. The latter possibility is favored by the inability of recombinant human SLPI to inhibit mouse PMN elastase while displaying potent inhibitory activity toward human PMN elastase in the same experiments (A. D., unpublished data).

SLPI: Possible Relation to the *Lps* Gene

In the virtual absence of LPS, the macrophage cell line of C3H/HeJ (Lps^{d}) origin overexpressed SLPI compared to the companion macrophage line from C3H/HeN (Lps^{n}) mice. On the other hand, primary macrophages and PMN from both strains expressed SLPI. Interpretation of the latter result is complicated by the fact that the cells were collected from mice after they were exposed to LPS as a contaminant in thioglycollate broth. Even cells from conventionally reared mice not injected with LPS can be expected to have encountered LPS through natural routes. Thus, SLPI may have been expressed for the most part constitutively in C3H/HeJ (Lps^{d}) phagocytes, but may have been induced by LPS in C3H/HeN (Lps^{n}) cells. Studies in axenic mice will be necessary to resolve this issue.

Cloning *SLPI* cDNA from a RAW 264.7 cell library allowed us to clone the open reading frame by RT-PCR

from C3H/HeJ and C3H/HeN cells, and establish that all three coding sequences are identical (F.-y. J., unpublished data). Moreover, our cloning of genomic mouse *SLPI* has permitted us to exclude its localization on chromosome 4 (A. D., unpublished data). Thus, SLPI is not the product of the *Lps* gene, but regulation of SLPI could be affected by allelic forms of *Lps*. Consistent with this speculation is that IFNγ corrects both the Lps^d-encoded defect and the LPS-inhibitory action of SLPI. That IFNγ suppresses expression of SLPI provides one possible explanation of the basis by which IFNγ normalizes responses to LPS in C3H/HeJ (Lps^d) mice.

SLPI: Regulation, Functions, and Mechanisms

SLPI is one of the few genes whose expression in macrophages is induced by LPS and suppressed by IFNγ. In human epithelial cells, *SLPI* was constitutively expressed and its expression was increased by phorbol ester, TNFα, and LPS at supraphysiologic concentrations (10 μg/ml) (Maruyama et al., 1994; Sallenave et al., 1994), as well as by synergistic combinations of elastase and corticosteroids (Abbinante-Nissen et al., 1993, 1995). Despite the cloning of the human *SLPI* gene, we are not aware of any studies of its promoter. Our cloning of the mouse *SLPI* promoter (A. D., unpublished data) will aid in understanding the unusual antagonistic relationship between LPS and IFNγ.

It remains to be determined by what mechanism SLPI inhibits macrophage responses to LPS. The proclivity of SLPI to undergo oxidative inactivation (Stolk et al., 1993) may explain why transfection with *SLPI* cDNA revealed its new biological actions, whereas no inhibition of LPS responses followed incubation of SLPI-nonproducing cells with SLPI producers or their conditioned medium (unpublished data).

Inhibition of LPS responses may be a consequence of SLPI's antiprotease action. This hypothesis is consistent with the findings that serine protease inhibitor diisopropylfluorophosphate blocked LPS-induced proliferation of B cells (Ku et al., 1981), while trypsin partially normalized this response in B cells from C3H/HeJ mice (Kuus-Reichel and Ulevitch, 1986). Moreover, treatment of human neutrophils or endothelial cells with urinary trypsin inhibitor, a 25 kDa stress-induced fragment of the plasma protein inter-α trypsin inhibitor, blocked the ability of LPS to trigger an elevation of intracellular Ca^{2+}; similar effects were seen with the isolated protease-inhibitory domain or with synthetic peptides encompassing the trypsin inhibitory site (Kanayama et al., 1995). Like SLPI, urinary trypsin inhibitor is active against neutrophil elastase and cathepsin G, as well as trypsin and chymotrypsin.

Alternatively, SLPI's action as an LPS inhibitor may be independent of its antiproteolytic function. Plasminogen activator inhibitor-1 (PAI-1), another serine protease inhibitor, blocked cell migration by nonproteolytically displacing cells' integrins from their attachment to vitronectin in the extracellular matrix (Deng et al., 1996; Stefansson and Lawrence, 1996). Integrins can influence macrophage responsiveness to LPS and can serve as cofactors for macrophage activation by other agents (Wright and Jong, 1986; Yurochko et al., 1992). When cross-linked by antibodies, integrins can even mimic IFNγ as a full macrophage-activating signal (Ding et al., 1987; Ingalls and Golenbock, 1995). Perhaps secreted SLPI, which can bind to some extracellular matrices (Kramps et al., 1989), blocks a costimulatory role served by integrins in macrophage responsiveness to LPS. Consistent with this speculation, our SLPI-expressing transfectants were less adherent than the SLPI-nonexpressing parental cells or control transfectants (Jin, F.-y. J., unpublished data).

Finally, SLPI may function by engaging a membrane receptor to induce an LPS-antagonistic signal. SLPI's three-dimensional structure suggests that it belongs to a protein family populated largely by neurotoxins that share equivalently placed loops held together by four similarly positioned disulfide bonds (Drenth et al., 1980). Where studied, these functionally divergent proteins all act by specific binding to membrane receptors. That SLPI may bind to macrophages is consonant with the view that salivary SLPI blocks infectivity of HIV by first binding to monocytes (McNeely et al., 1995).

The inhibitory effect of SLPI on LPS-induced secretion of NO and TNFα is likely to reflect interference with early steps of signaling. NF-κB/rel binding site(s) serve as LPS-response elements in the promoters of many LPS-inducible genes, including the two whose products are monitored here, i.e., inducible NO synthase and TNFα (Xie et al., 1994; Sweet and Hume, 1996). Activation of the NF-κB complex was inhibited in SLPI-overexpressing GG2EE (Lps^d) cells and SLPI-transfected HeNC2 (Lps^n) cells.

SLPI accounts for 80%–90% of the elastase-inhibitory capacity of bronchial secretions (Tegner, 1978). Administration of SLPI can protect animals from emphysema induced by neutrophil elastase or by LPS (Lucey et al., 1990; Rudolphus et al., 1993). LPS-induced expression of SLPI in PMN suggests the existence of a feedback mechanism in inflammatory reactions, whereby LPS, a major stimulus for recruitment of PMN, elicits from PMN an inhibitor of some of the major tissue-damaging enzymes that they import.

Identification of SLPI as an LPS-induced LPS inhibitor raises the possibility that SLPI may be involved in the phenomenon of LPS tolerance; that is, the ability of an initial exposure to LPS to cause relative refractoriness to a subsequent exposure (Beeson, 1947). Other candidates to mediate LPS tolerance include glucocorticoids (Beutler et al., 1986a; Hogan and Vogel, 1988), IL-10 (Bogdan et al., 1991; Randow et al., 1995), and TGF-β (Ding et al., 1990; Randow, et al., 1995). A role for SLPI as a mediator of glucocorticoid action in LPS tolerance can be envisioned, since tolerogenic doses of LPS elevate plasma glucocorticoids, and glucocorticoids increased human *SLPI* transcripts in human airway epithelial cells (Abbinante-Nissen et al., 1995). If plasma levels of SLPI increase in septic states, as our findings would predict, then SLPI may be one of the unidentified factors in septic shock patients' plasma that suppresses monocyte responsiveness to LPS (Brandtzaeg et al., 1996).

Experimental Procedures

Materials

Reagents and supplies were obtained as follows: LPS prepared by phenol extraction from List Biological Laboratories (Campbell, CA);

mouse IFNγ (protein concentration 1.1 mg/ml; sp. act. 5.2×10^6 U/mg; LPS content <10 pg/ml) from Genentech (South San Francisco, CA); oligonucleotide primers from Oligo Etc., Inc. (Guilford, CT); G418, reverse transcription buffer, and Moloney murine leukemia virus reverse transcriptase from Gibco Life Technologies (Grand Island, NY); restriction enzymes from New England Biolabs (Beverly MA); AmpliTaq DNA polymerase, dNTPs, and PCR buffer solutions from Perkin Elmer Cetus (Foster City, CA); guanidium isothiocyanate, formaldehyde, and formamide from Fluka Chemica-Biochemica (Ronkonkoma, NY); and all other reagents from Sigma (St. Louis, MO) except as specified below. Tissue culture dishes were from Corning Glass Works (Corning, NY).

Mice

Charles River Breeding Laboratories (Wilmington, MA) and Jackson Laboratories (Bar Harbor, ME) supplied C3H/HeN (*Lps*^{n}) and C3H/HeJ (*Lps*^{d}) female mice, respectively.

Cells

Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine, 200 units/ml penicillin, and 200 μg/ml streptomycin at 37°C in 5% CO_2/95% air. Complete culture medium was routinely monitored for LPS contamination by the chromogenic limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD), and found to contain <25 pg LPS/ml. ANA-1 cells were kindly provided by Dr. L. Varesio (NCI, Frederick, MD). ANA-1, HeNC2, and GG2EE cells are bone marrow-derived J2 virus-transformed macrophage cell lines, according to the method described previously (Blasi et al., 1987; Cox et al., 1989). J774.1 was provided by D. Falcone (Cornell University Medical College, NY). HeNC2 cells stably transfected with p463-neo-SLPI or p463-neo vectors were maintained in 500 μg/ml G418. RAW 264.7 cells were from American Type Culture Collection (Rockville, MD). Primary mouse macrophages and PMN were collected from the peritoneal cavity 4 and 0.5 days, respectively, after intraperitoneal injection with 2 ml of 4% Brewer's thioglycollate broth (DIFCO, Detroit, MI). Spleens from the mice were minced and triturated through an 18-gauge needle. Nonadherent splenocytes were collected after 2 hr culture in flat vessels to deplete adherent cells.

Differential Display

Cultured HeNC2 (*Lps*^{n}) and GG2EE (*Lps*^{d}) cells were lysed with 4 M guanidinium isothiocyanate. The cell lysate was layered on 5.7 M CsCl, 0.1 M EDTA, and centrifuged at 35,000 × g at 4°C for 14 hr in an SW-40 rotor in an L8-M ultracentrifuge (Beckman, Palo Alto, CA). The RNA pellet was dissolved in 0.1% diethyl pyrocarbonate-treated H_2O and subjected to digestion with DNase I (1 unit/ml, RNase-free) (GIBCO Life Technologies, Grand Island, NY) for 30 min at 37°C. Digested RNA was extracted with phenol/chloroform and precipitated with ethanol.

For differential display (Liang et al., 1993), 1 μg DNA-free total RNA was used for the reverse transcription reaction (final volume, 20 μl) with 20 μM dNTPs, 0.8 u/μl RNAsin (Promega), and 1 mM primer T_{12}MC at 65°C, 5 min at 37°C, 60 min at 95°C, and 5 min at 4°C. Reverse transcriptase (100 units) was added after 10 min incubation at 37°C. PCR reaction was performed (final volume, 20 μl) with PCR buffer II, 2.5 mM $MgCl_2$, 2 μM dNTPs, 0.2 μM primer ACCATGGACT, 1 μM T_{12}MC, 2 μl reverse transcription mix, 1 μl 35S-dATP (1200 Ci/mmol) (NEM Life Science Products, Boston, MA), and 1 unit AmpliTaq polymerase at 94°C, 30 s at 40°C, 2 min at 72°C, 30 s for 40 cycles at 72°C, and 5 min at 4°C. Samples (4 μl) were run on 6% sequencing gels. Differentially expressed products were extracted with H_2O and reamplified twice in the PCR reaction using the same set of primers. The reamplified PCR product was subcloned into PCR AT cloning vector (Invitrogen, San Diego, CA), and sequenced.

Northern Blot

20 μg of total RNA was run on a 1% agarose gel with 0.2 M 3-[N-morpholino] propanesulfonic acid (pH 7.0), 0.5 M sodium acetate, 10 mM EDTA (1 × MOPS), and 2% formaldehyde, confirming equal loading by means of staining with 2.5 μg/ml ethidium bromide. RNA was transferred in 20 × SSC onto nylon membrane (NEN) (Research Products, Boston, MA). The membrane was hybridized for 18 hr at 42°C with labeled probe (10^6 cpm/ml) in 5 × SSC, 5 × Denhart buffer, 50% formamide, and 1% SDS plus 100 μg/ml of sperm DNA. Membranes were then washed twice with 1 × SSC, 0.1% SDS (10 min, room temperature), and with 0.25 × SSC, 0.1% SDS (10 min, 55°C) before autoradiography.

cDNA Cloning

Subcloned cDNA fragment from differential display was used as a probe to screen for full-length cDNA from a customized RAW 264.7 macrophage cDNA library in a lambda phage ZAP vector (Stratagene, La Jolla, CA) according to the instruction manual provided by the supplier.

Generation of Antibody to Recombinant Protein

A *SLPI* cDNA fragment (BamHI–XhoI: 495–1123) coding for the last ten amino acids of the signal peptide and full-length secreted SLPI was subcloned into pQE32 vector (Qiagen, Chasworth, CA) for a fusion protein with hexahistidine attached to the N-terminus. M15 cells were transformed with this plasmid and grown in 1 l LB at 37°C with ampicillin (100 μg/ml) and kanamycin (25 μg/ml) to $OD_{600} = 0.7$. The bacterial pellet was collected 4 hr after IPTG (1 mM) induction and sonicated in 20 ml of sonication buffer (50 mM Na-phosphate [pH 8.0], 300 mM NaCl, 1 mg/ml lysozyme). The pellet (13,000 × g, 30 min) was resuspended in 20 ml of denaturing buffer (6 M guanidinium HCl, 0.1 M Na-phosphate, 0.01 M Tris-HCl [pH 8.0], 10 mM β-mercaptoethanol) for 1 hr at room temperature. The supernate (10,000 × g, 15 min at 4°C) was stirred for 60 min at room temperature with 5 ml of 50% slurry of Ni^+-NTA resin, and the resin was washed with 10 vol denaturing buffer and 10 vol of washing buffer I (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl [pH 8.0], 10 mM β-mercaptoethanol), followed by washing buffer II (washing buffer I [pH 6.3]) until OD_{280} of the wash was <0.01. Recombinant protein was eluted with 10 ml of washing buffer II plus 250 mM imidazole and sequenced by Edman degradation (Microbiological Associates, Rockville, MD) to confirm identity and purity. SDS-gel slices containing 100 μg purified protein were emulsified with Freund's complete adjuvant for injection in rabbits.

Immunoprecipitation and Western Blot

Cells were cultured for two days at 10^6 per 2 ml per 36 mm–diam well. Anti-mouse SLPI antiserum (1:1000) and immobilized protein A beads (Pierce, Rockford, IL) were incubated with the collected medium overnight at 4°C. Beads were washed with buffer TBS (25 mM Tris [pH 7.5], 150 mM NaCl) with 0.5% NP40, 0.5% Triton X-100, 1.5 mM $MgCl_2$, and 10% glycerol, followed by TBS. Immunoprecipitates were boiled for 5 min in reducing SDS-PAGE sample buffer and subjected to SDS-PAGE. Proteins were transferred to a 0.20 μm–pore nitrocellulose membrane (Schleicher and Shuell, Inc., Keene, NH) in 20% methanol, 25 mM Tris, and 192 mM glycine (pH 8.3). The membrane was blocked with 5% milk and blotted with anti-mouse SLPI antiserum (1:1,000), followed by goat anti-rabbit-IgG coupled to horseradish peroxidase (1:10,000) (Amersham, Arlington Hights, IL). The bound antibody was detected by ECL (NEN Research Products, Boston, MA).

Assays for Secretion of NO_2^- and TNFα

Cells were plated in 96-well plates at 10^5 cells per well in 100 μl of medium and treated 24 hr or 48 hr with indicated concentrations of LPS, IFNγ, or both. Conditioned medium (100 μl) was mixed with equal volume of Greiss's reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4). Absorbance at 550 nm was recorded in a microplate reader (MR5000, Dynatech, Chantilly, VA) with sodium nitrite standards. Nitrite content of similarly incubated cell-free medium was subtracted. For TNFα ELISA (Duoset, Genzyme, Cambridge, MA), culture supernatant was collected at 12 hr or 24 hr and tested according to the manufacturer's instructions.

Generation of Stable Transfectants

p463-Neo, a pUC19-based vector, was generated by combining the spleen focus-forming virus LTR from pFNeo with the BamHI to EcoRI fragment of the human growth hormone. The hGH sequences and

polyadenylation signals were added to increase mRNA stability for cDNA expression (Costa et al., 1992). The vector was ligated with mouse *SLPI* cDNA EcI136III-NaeI fragment (435 bp including open reading frame) to generate the plasmid p463-Neo-SLPI. Plasmid p463-Neo-SLPI and the vector control plasmid p463-Neo were transfected into HeNC2 (*Lps*^{n}) cells using N-[1-(2, 3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DODAP, Boehringer Mannheim). After two weeks of selection in G418 (500 μg/ml), expanded cells were subjected to limiting dilution in the presence of feeder cells (murine resident peritoneal cells) to select individual stable transfectants.

Electrophoretic Mobility Shift Assays (EMSA)

Cells were treated with LPS or IFNγ. NF-κB or GRR binding activities in the nuclear extracts were determined as described (Pearse et al., 1991; Xie et al., 1994).

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GenBank Accession Number

The GenBank accession number for mouse SLPI is U73004 and the GenBank accession number for human SLPI is X04502.