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Ablation of Leptin Receptor-Mediated ERK Activation Impairs Host Defense against Gram-Negative Pneumonia

Peter Mancuso,*† Martin G. Myers, Jr.,‡§,* Deepti Goel,* Carlos H. Serezani,‖ Edmund O’Brien,*# Jared Goldberg,* David M. Aronoff,†‡,§,** and Marc Peters-Golden †‡,§,*††

The adipo-cytokine-derived hormone leptin plays an important role in regulation of energy homeostasis and the innate immune response against bacterial infections. Leptin’s actions are mediated by signaling events initiated by phosphorylation of tyrosine residues on the long form of the leptin receptor. We recently reported that disruption of leptin receptor-mediated STAT3 activation augmented host defense against pneumococcal pneumonia. In this report, we assessed leptin receptor-mediated ERK activation, a pathway that was ablated in the /l mouse through a mutation of the tyrosine 985 residue in the leptin receptor, to determine its role in host defense against bacterial pneumonia in vivo and in alveolar macrophage (AM) antibacterial functions in vitro. /l mice exhibited increased mortality and impaired pulmonary bacterial clearance after intratracheal challenge with Klebsiella pneumoniae. The synthesis of cysteinyI-leukotrienes was reduced and that of PGE2 enhanced in AMs in vitro and the lungs of /l mice after infection with K. pneumoniae in vivo. We also observed reduced phagocytosis and killing of K. pneumoniae in AMs from /l mice that was associated with reduced reactive oxygen intermediate production in vitro. cAMP, known to suppress phagocytosis, bactericidal capacity, and reactive oxygen intermediate production, was also increased 2-fold in AMs from /l mice. Pharmacologic blockade of PGE2 synthesis reduced cAMP levels and overcame the defective phagocytosis and killing of bacteria in AMs from /l mice in vitro. These results demonstrate that leptin receptor-mediated ERK activation plays an essential role in host defense against bacterial pneumonia and in leukocyte antibacterial effector functions. The Journal of Immunology, 2012, 189: 000–000.

Pneumonia is a common consequence of malnutrition, a leading threat to human health throughout the world regardless of socioeconomic status (1). Rapid depletion of energy storage in the form of adipose tissue often occurs during periods of famine in the developing world and in hospitalized patients suffering from chronic and critical illness (2–6). Associated with the decline in fat mass is a decrease in leptin, an adipokine produced by white adipose tissue and known to regulate energy homeostasis. Under normal circumstances, leptin levels are correlated with adipose tissue mass (7). However, during acute bacterial infections and after endotoxin administration in laboratory animals, leptin levels increase disproportionately to fat mass (8–12). An important role for leptin in the regulation of immune function during periods of fasting, obesity, and in disease states mediated by inflammation is emerging.

We and others have observed that leptin plays a protective role in the host response against infectious disease (13–18). Using murine models of Klebsiella and pneumococcal pneumonia, we have found that leptin deficiency induced by genetic means or by fasting compromised pulmonary bacterial clearance and survival. This defect in pulmonary host defense was associated with abrogated alveolar macrophage (AM) and polymorphonuclear neutrophil (PMN) phagocytosis and killing of bacteria in vitro (12–14, 19). The mechanisms underlying defective leukocyte effector function in cells from leptin-deficient mice were associated with a reduction in leukotriene (LT) synthesis in AMs, reduced complement receptor (CR3) expression, and decreased H2O2 synthesis in PMNs (12, 14, 19). Other studies have revealed that the production of cytokines IL-6, MIP-2, and MCP-1 in leptin-deficient or leptin receptor-deficient mice was lower than that observed for wild-type animals (13, 15). The intracellular signaling events downstream of the leptin receptor (LepR) that regulate leukocyte effector functions, in the context of bacterial pneumonia, have not been determined.

LepR signaling is mediated by the long isoform of the leptin receptor (LepRβ) via the JAK–STAT and MAPK signaling pathways. Upon binding to its ligand (Fig. 1), the LepRβ activates the constitutively associated JAK2 tyrosine kinase to induce tyrosine phosphorylation-dependent signaling via several divergent pathways. JAK2 mediates phosphorylation of Tyr1138, which binds and mediates the phosphorylation-dependent activation of the latent transcription factor, STAT3. After nuclear translocation, STAT3 activates transcription of SOCS-3, a protein that inhibits JAK2 and STAT3 signaling during prolonged stimulation of the LepRβ (20). LepRβ-mediated phosphorylation of Tyr1077 activates STAT5.

*Department of Environmental Health Sciences, School of Public Health, University of Michigan, Ann Arbor, MI 48109; †Graduate Program in Immunology, University of Michigan, Ann Arbor, MI 48109; ‡Division of Molecular Endocrinology and Diabetes, University of Michigan School of Medicine, Ann Arbor, MI 48109; †Department of Integrative Physiology, School of Medicine, University of Michigan, Ann Arbor, MI 48109; ‡Division of Internal Medicine, University of Michigan, Ann Arbor, MI 48109; †Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN 46202; Toxicology Program, School of Public Health, University of Michigan, Ann Arbor, MI 48109; **Division of Infectious Diseases, University of Michigan, Ann Arbor, MI 48109; and ††Division of Pulmonary and Critical Care Medicine, University of Michigan, Ann Arbor, MI 48109.

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Address correspondence and reprint requests to Dr. Peter Mancuso, Department of Environmental Health Sciences, School of Public Health, University of Michigan, 6627 SPPHA, Ann Arbor, MI 48109. E-mail address: pmancuso@umich.edu

Abbreviations used in this article: AM, alveolar macrophage; BAL, bronchoalveolar lavage; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; cysLT, cysteinyl-leukotriene; GRB2, growth factor binding 2; LepR, leptin receptor; LepRβ, long isoform of the leptin receptor; 5-LO, 5-lipoxygenase; LT, leukotriene; LTB4, leukotriene B4; MOI, multiplicity of infection; mPGEs-1, microsomal prostaglandin E synthase-1; Pen/Strep, penicillin–streptomycin; PMN, polymorphonuclear neutrophil; RFU, relative fluorescence unit; ROI, reactive oxygen intermediate; SHP-2, SH2-containing tyrosine phosphatase; WT, wild-type.

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signaling (21). Finally, phosphorylation of Tyr\textsuperscript{985} recruits binding partners SH2-containing tyrosine phosphatase (SHP-2) and growth factor binding 2 (GRB2), which activate ERK1/2 (22). The generation of the l/l mouse was first described by Björnholm et al. (23), who reported that these animals lack the ability to activate the ERK1/2 pathway via the LeprR due to a substitution mutation at Tyr\textsuperscript{985} with L\textsuperscript{985}. Although a recent report by Guo et al. (17) demonstrated that l/l mice exhibit greater susceptibility to enteric Entamoeba histolytica infection, the mechanism responsible for this defect is unknown, and bacterial infections have not been studied in these mice.

The role of Lepr-mediated (Lepr\textsuperscript{->}) signaling events in the innate immune response against bacterial infections is complex and difficult to study in vivo. Not all Lepr mutations result in impaired immunity. For example, we recently reported that disruption of Lepr-mediated STAT3 signaling improved AM phagocytosis and killing of bacteria in vitro and host defense against pneumococcal pneumonia in s/s mice in vivo (18). In the current report, we assessed the contribution of intracellular signals initiated by the Lepr\textsuperscript{->}Tyr\textsuperscript{985} by comparing the responses of wild-type (WT) and l/l mice in a murine model of bacterial pneumonia. We demonstrate for the first time, to our knowledge, that l/l mice exhibit increased susceptibility to Gram-negative pneumonia and that this pathway plays an essential role in the innate immune response against bacterial pneumonia.

Materials and Methods

Animals

Heterozygous CBYL6 mice were intercrossed in the University of Michigan Institute for Laboratory Animal Medicine (Ann Arbor, MI) to generate age- and gender-matched male and female (Lep\textsuperscript{+/->}Lep\textsuperscript{+/->}) l/l and WT (+/+) littermates 8–14 wk of age (23). Animals were genotyped by TaqMan SNP allelic discrimination assays and were treated according to National Institutes of Health guidelines for the use of experimental animals with the approval of the University of Michigan Committee for the Use and Care of Animals.

Cell isolation and culture

Resident AMs were recovered from mice by bronchoalveolar lavage (BAL) as previously described, resuspended in RPMI 1640 (Life Technologies, Invitrogen, Carlsbad, CA) to a concentration of 2 × 10\textsuperscript{6} cells per milliliter, and allowed to adhere to tissue-culture plates for 1 h (37°C, 5% CO\textsubscript{2}) (24). After replacing the media with RPMI 1640 containing 10% FBS (Life Technologies, Invitrogen) and 1% penicillin–streptomycin (Pen/Strep) (Invitrogen), the cells were cultured overnight. PMNs were obtained from mice by peritoneal lavage 5 h after an i.p. injection of a 1% glycerol solution in PBS as previously described (25).

Immunoblot analysis

AMs obtained from WT and l/l mice were plated at 4 × 10\textsuperscript{6} cells per well and cultured overnight in RPMI 1640 containing FBS and Pen/Strep. On the following day, the cells were prepared for lysis or cultured with nontreated or leptin (50 ng/ml) for 5, 15, 30, or 60 min for the assessment of ERK activation. The macrophages were then washed with HBSS and scraped with ice-cold lysis buffer (RIPA buffer; Sigma), and cells were disrupted with sonication (10 bursts at 20% duty cycle). Twenty micrograms of protein, as determined by a modified Coomassie blue binding assay ( Pierce Chemical, Rockford, IL), was separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Membranes were probed with the rabbit anti-IL-1 (Cayman Chemical, Ann Arbor, MI) and actin (American Research Products, McGraw Park, IL) antibodies, was separated by SDS-PAGE under reducing conditions and transferred by a modified Coomassie blue binding assay (Pierce Chemical, Rockford, IL). The densities of the luminescent bands were quantitated in appropriately exposed nitrocellulose by using Image Reader (Fuji Film). The density value of the p-ERK1/2, 5-LO, COX-1, COX-2, and mPGES-1 bands were divided by the density value of the ERK1/2 (p-ERK1/2) or GAPDH (5-LO, COX-1, COX-2, and mPGES-1) bands, respectively, to normalize the relative band densities.

Klebsiella pneumoniae preparation and inoculation

K. pneumoniae strain 43816, serotype 2, was obtained from the American Type Culture Collection (Manassas, VA), and aliquots were grown in tryptic soy broth (Difco, Detroit, MI) for 18 h at 37°C. The concentration of bacteria in culture was determined spectrophotometrically (A\textsubscript{660}). K. pneumoniae was then pelleted by centrifugation (13,000 rpm for 3 min) two times, resuspended in PBS, and serially diluted in PBS to obtain the appropriate concentration. Mice were anesthetized with ketamine and xylazine as previously described (12). A midline incision was made to expose the trachea, a 30-μl inoculum containing 5 × 10\textsuperscript{7} CFU K. pneumoniae was administered via the trachea using a 26-gauge needle, and the wound was closed using surgical glue (Nexaband, Phoenix, AZ) (12).

Determination of survival and lung and spleen K. pneumoniae CFUs

After intratracheal inoculation with K. pneumoniae, mice were evaluated for survival daily for 7 d. Four and twenty-four hours after K. pneumoniae challenge, mice were euthanized by CO\textsubscript{2} asphyxiation, and lungs and spleen were harvested. CFU determinations were performed using commercially available EIA kits according to the manufacturer’s instructions (26). Briefly, lungs and spleen were homogenized in 0.5 ml of sterile saline, serially diluted, and plated on soy-based blood agar plates (Difco). After 18 h at room temperature, CFUs were enumerated.

Blood and lung leukocyte differential and total cell count

Four and twenty-four hours postinfection, lung leukocytes were obtained from mice by BAL after CO\textsubscript{2} asphyxiation, and differential counts were performed on cells after staining with a modified Wright-Giemsa stain (American Scientific Products, McGraw Park, IL). Blood was collected by cardiac puncture for peripheral blood cell counts using a Hemavet cell analyzer (Drew Scientific) operated by the University of Michigan Institute for Laboratory Animal Medicine Animal Diagnostic Laboratory.

Determination of cytokines, cysteinyl-leukotrienes, leukotriene B\textsubscript{4}, PGE\textsubscript{2}, and leptin

In a separate group of mice, lungs obtained from euthanized mice 4 and 24 h postinfection were homogenized, and cytokine [CXCL2 (MIP-2), CCL2 (MCP-1), IL-6, IL-10, IL-12, and TNF-α] (R&D Duoset, R&D Systems, Minneapolis, MN) and cysteinyl-leukotrienes (cyst-LTs), leukotriene B\textsubscript{4} (LTB\textsubscript{4}), and PGE\textsubscript{2} (Cayman Chemical, Ann Arbor, MI) levels were determined using commercially available EIA kits according to the manufacturer’s instructions. Blood leptin levels were determined using an EIA kit from R&D Systems according to the manufacturer’s instructions.

Fluorometric assay of AM phagocytosis

AM phagocytosis of K. pneumoniae was assessed using a previously published protocol for determining the ingestion of fluorescent, FITC-labeled Streptococcus pneumoniae (24). Briefly, AMs obtained by BAL were adhered and seeded in replicates of eight to 384-well tissue culture plates with opaque sides and optically clear bottoms (Costar, Corning Life Sciences, Lowell, MA) and cultured overnight with Hemul 1640 with 1% Pen/Strep and 10% FCS (Invitrogen). On the following day, FITC-labeled K. pneumoniae were opsonized with 3% immune serum as previously described (27). AMs pretreated with RPMI 1640 media alone, with indomethacin (10 μM) (Cayman Chemical) for 30 min, or with cyst-LTs (100 nM) and LTB\textsubscript{4} (1 μM) (alone or together) for 15 min were incubated with opsonized FITC- K. pneumoniae using a multiplicity of infection (MOI) of 150:1 for 60 min to allow phagocytosis to occur. Trypan blue (250 μg/ml; Molecular Probes) was added for 1 min to quench the fluorescence of extracellular bacteria, and fluorescence was determined using a Spectrmax Gemini EM fluorometer with 485-nm excitation/535-nm emission (Molecular Devices, Sunnyvale, CA). The phagocytic index was calculated as previously described in relative fluorescence units (RFUs) (18, 24). Three separate experiments were conducted with eight replicate wells for every experimental condition, and the RFUs were normalized to the control condition (untreated AMs from WT animals) in each experiment.

Bacterial assays

The survival of internalized K. pneumoniae within the AM was quantified using a tetrazolium dye reduction assay, as described previously (27).
Briefly, 2 × 10^5/ml AMs, prepared as described previously, were adhered in quintuplicate in 96-well, half-area, tissue culture dishes (Corning, Lowell, MA). After overnight culture, K. pneumoniae were opsonized with 3% anti-K. pneumoniae rat specific immune serum, as previously described (28). Cells were then treated with either cell culture media alone or indomethacin (10 μM) for 30 min and were infected with an 0.1-ml suspension of opsonized K. pneumoniae (1 × 10^7 CFU/ml; MOI, 50:1) for 30 min to allow phagocytosis to occur. The AMs were then washed three times with PBS to remove extracellular bacteria and incubated for an additional 60 min to permit intracellular killing. The remainder of the assay was completed, as described elsewhere (27). On the basis of this assay, it has been determined that the intensity of the absorbance at 595 nm is directly proportional to the number of intracellular bacteria associated with the macrophages. Results were expressed as percentage of survival of ingested bacteria, where the survival of ingested bacteria = 100% × A595 control plate/A595 experimental plate.

Reactive oxygen intermediate production

AMs were adhered to 384-well plates at a concentration of 1.25 × 10^5 cells/well and cultured overnight in RPMI 1640 containing 10% FCS and antibiotics. On the next day, the medium was replaced with PBS containing 10 μM H2DCF, and the cells were cultured for 1 h. The medium was then replaced with warmed HBSS, and the cells were stimulated with heat-killed K. pneumoniae opsonized with 3% specific immune serum using an MOI of 50:1. Reactive oxygen intermediate (ROI) production was assessed every 30 min for 2 h by measuring fluorescence using a Spectramax Gemini XS fluorometer (Molecular Devices) with excitation/emission setting at 493/522 nm.

Assessment of NO production

AMs were adhered to 96-well plates at a concentration of 2 × 10^5 cells/well and cultured with DMEM supplemented with 1% sodium pyruvate (Invitrogen) containing 10% FCS and penicillin-streptomycin with or without 10 ng/ml LPS from E. coli (Sigma-Aldrich) and 10 ng/ml IFN-γ (R&D Systems) for 24 h. NO production was determined by measuring stable nitrite (NO_2^-) concentrations using a modified Griess reaction with a commercially available assay kit according to the manufacturer’s instructions (Cayman Chemical).

Measurement of intracellular cAMP by AMs in vitro

AMs were cultured overnight in 96-well plates in RPMI 1640 with 10% FCS and 1% penicillin-streptomycin at concentrations of 2 × 10^5 cells/well. On the following day, the cell culture media was replaced with warm RPMI 1640, and AMs were incubated for 30 min in the presence or absence of indomethacin (10 μM) with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (EMD Biosciences) for 30 min prior to stimulation with heat-killed K. pneumoniae opsonized with 3% rat specific immune serum using an MOI of 50:1. After 1 h, culture supernatants were aspirated and the cells were lysed by incubation for 20 min with 0.1 M HCl (22°C) for cAMP experiments. The cells were then disrupted using a cell scraper, and intracellular cAMP levels were determined by ELISA kit according to the manufacturer (Cayman Chemicals).

Statistical analyses

Where appropriate, mean values were compared using a paired Student t test, a one-way or a two-way ANOVA followed by the Bonferroni correction. Survival was evaluated for differences using a log-rank test. Differences were considered significant if p ≤ 0.05, and the actual p values are mentioned in the results section. All experiments were performed on at least three separate occasions unless otherwise specified. Data are presented as mean values ± SEM unless otherwise noted.

Results

Substitution of LepRb Tyr^{985} with L985 in l/l mice abrogates LepRb-mediated ERK1/2 activation

To confirm that l/l mice lack the ability to signal via LepRb Tyr^{985}, we assessed ERK1/2 activation using immunoblot analysis of p-ERK1/2 in AMs obtained from WT and l/l mice cultured with leptin. As shown in Fig. 1C, levels of total ERK1/2 were the same for both groups of mice. However, when AMs from WT mice were cultured with exogenous leptin for 30 min, we observed an increase in p-ERK1/2 as determined by a 50% increase in p-ERK1/2 (p = 0.0002). We conducted time-course experiments for ERK activation (p-ERK) (i.e., 5, 15, and 30 min after stimulation with leptin), and only the blots from cells stimulated for 30 min are shown as this represents the peak of this response. In contrast, we did not observe any increases in p-ERK1/2 levels in AMs from l/l mice after leptin treatment for 30 min or at any other time point (p = 0.12). Other signaling events initiated by this mutant receptor such as LepRb→STAT3 or STAT5 are normal as previously reported (21, 23). In addition, hypothalamic p-ERK activation was not observed in a previous report using l/l mice treated with much higher doses of leptin (5 μg/g of body weight) (23). Blood leptin levels were slightly lower (p = 0.04) in the l/l mice (2.9 ± 0.5 ng/ml) compared with that of WT animals (4.5 ± 0.9 ng/ml) as previously reported (23). These data indicate that leptin induces phosphorylation of ERK1/2 via the LepRb Tyr^{985} and that this pathway is abrogated in AMs from l/l mice.

l/l mice exhibit greater mortality and reduced pulmonary bacterial clearance after K. pneumoniae challenge

We have previously demonstrated that ob/ob mice, which lack functional leptin, or mice rendered leptin deficient by fasting are more susceptible to both Gram-negative and Gram-positive pneumonia (12, 13). To determine if intracellular signals arising from the LepRb Tyr^{985} play a role in pulmonary host defense against Gram-negative pneumonia, we compared the responses of WT and l/l mice after an intratracheal challenge with K. pneumoniae. As shown in Fig. 2A, l/l mice exhibited substantially lower survival (27%) compared with that of WT (65%, p = 0.04) after K. pneumoniae challenge 7 d postinfection. Because the differences in survival may indicate impaired pulmonary host defense in l/l mice, we assessed the bacterial burdens in the lungs and spleen of mice 4 and 24 h postinfection. We chose these time points because we observed that the first death recorded for an l/l mouse occurred 48 h after K. pneumoniae challenge. As shown in Fig. 2B, bacterial burdens were ~1-log fold greater after 4 h (p = 0.02) and 4-log fold higher at 24 h (p = 0.0001) in l/l compared with WT animals. We did not find any bacterial CFUs in spleens harvested from any of these animals 4 h (p = 0.38) and 24 h (p = 0.73) postinfection. These results indicate a defective pulmonary innate immune response against pulmonary bacterial infection in l/l mice.

Modest differences between WT and l/l mice in leukocyte recruitment after K. pneumoniae challenge

The recruitment of leukocytes to the lungs is essential for effective host defense during bacterial pneumonia. We have previously observed increased PMN recruitment to the lungs of leptin-deficient (ob/ob) mice and attenuated leukocyte recruitment in mice rendered leptin deficient by fasting in response to pneumococcal pneumonia (12, 13). To determine if the ablation of LepRb Tyr^{985} signaling alters leukocyte recruitment to the lung during bacterial pneumonia, we recovered leukocytes by lavage in mice 4 and 24 h postinfection as these were the time points when we observed differences in pulmonary bacterial burdens. As shown in Fig. 3, there were no differences (p = 0.29) between WT and l/l mice in total or differential leukocyte counts in BAL fluid 4 h after K. pneumoniae challenge. However, we did find lower BAL fluid monocyte/macrophage (p = 0.04) and higher neutrophil (p = 0.04) counts in l/l mice at this time point. We also observed higher total peripheral blood leukocyte counts (l/l 6.7 × 10^6 WBC/ml versus WT 4.1 × 10^6 WBC/ml) (p = 0.04) and elevated PMN counts (l/l 3.8 × 10^5 versus WT 1.9 × 10^5) (p = 0.04) in the l/l mice 24 h postinfection. These results indicate that differences in PMN recruitment, known to play a critical role in host defense against Gram-negative pneumonia (29), do not explain the limitations in Klebsiella clearance from the lungs of l/l mice.
Pulmonary cytokine and eicosanoid levels after K. pneumoniae challenge

To determine whether the impairment in pulmonary bacterial clearance was due to differences in proinflammatory mediators produced in the lung during infection, we assessed lung homogenate cysLTs, PGE2, and cytokine levels after bacterial challenge. As shown in Fig. 4A, there were no differences in lung homogenate cytokines known to play an important role in antibacterial host defense (IL-6, TNF-α, IL-10, IL-12, MIP-2, TNF-α) 4 h postinfection. However, we did find major differences in lipid mediators. Cyclooxygenase-derived PGE2 was elevated ($p = 0.02$), whereas the 5-LO–derived cysLTs were reduced ($p = 0.01$) in l/l mice at this time point. Reduced cysLT levels persisted 24 h postinfection in the lungs of l/l mice ($p = 0.02$) (Fig. 4C), whereas no differences in PGE2 or cytokines were observed ($p = 0.93$) (Fig. 4D).

Impaired phagocytosis, killing, and ROI production in AMs from l/l mice

The observed 1-log fold elevation of bacterial CFUs in l/l mice 4 h postinfection suggests a defect in the pulmonary innate immune response and implicates the resident AM, which plays a critical role in the early stages of K. pneumoniae clearance. To test this possibility, we compared the ability of cells from WT and l/l mice to phagocytose and kill opsonized K. pneumoniae in vitro. As shown in Fig. 5A, phagocytosis of K. pneumoniae opsonized with immune serum was 25% less in AMs from l/l mice than that observed in cells from WT animals ($p = 0.03$). Next, we asked if there were differences in the ability of AMs from WT and l/l mice...
to kill ingested *K. pneumoniae*. As shown in Fig. 5B, we observed that AMs from WT mice were able to kill 40% of the bacteria that had been phagocytosed during the allotted interval. In contrast, AMs from *l/l* mice were able to kill less than 10% of the phagocytosed bacteria during this interval, suggesting a severe defect in AM effector function (WT versus *l/l*, *p* = 0.04). Previously, we had demonstrated that ROI production in neutrophils from *ob/ob* mice stimulated with *S. pneumoniae* was reduced (14). In the current study, we observed that ROI production was reduced in AMs from *l/l* mice compared with that of WT animals by ∼40% 120 min after stimulation with opsonized *K. pneumoniae* (*p* = 0.0008). Likewise, we also observed a similar reduction in ROI production in glycogen-elicited PMNs obtained from *l/l* mice (*p* = 0.01) (data not shown). We did not, however, find differences in AM NO production after stimulation overnight with LPS and IFN-γ (*p* = 0.15) (data not shown). These results indicate that the LepRb→Tyr985 mutation in *l/l* mice impairs AM phagocytosis and killing of ingested bacteria and reduces the ability of AMs and PMNs to generate ROI in vitro.

**FIGURE 3.** Leukocyte recruitment in WT and *l/l* mice after intratracheal *K. pneumoniae* infection. WT (open bars) and *l/l* (solid bars) mice were infected via the intratracheal route with 5 × 10³ CFU *K. pneumoniae*. Four (A) and twenty-four (B) hours later, cells were recovered by BAL, and the total differential leukocyte, monocytes/macrophages (Mono/Mac), and neutrophil (PMN) counts were determined as described in *Materials and Methods*. Bars represent the mean ± SEM of *n* = 10 mice per group. *p* < 0.05 (compared with WT using a Student *t* test).

**FIGURE 4.** Elevated PGE₂ and reduced cysLTs in *l/l* mice in lung homogenates after intratracheal *K. pneumoniae* challenge. WT (open bars) and *l/l* (solid bars) mice were infected via the intratracheal route with *K. pneumoniae*. Lung homogenates were prepared from mice 4 h (A, B) and 24 h (C, D) after intratracheal bacterial challenge, and eicosanoids (cysLTs and PGE₂) and cytokines (IL-6, IL-10, IL-12, MIP-2, and TNF-α) were determined as mentioned in *Materials and Methods*. Bars represent the mean ± SEM of *n* = 5–10 mice per group. *p* < 0.05 (compared with WT levels at 4 and 24 h postinfection using a Student *t* test).

**Diminished LTs and enhanced PGE₂ production explained by reduced 5-LO and increased mPGEs-1 expression in AMs from *l/l* mice**

The ability of AMs to phagocytose and kill bacteria is enhanced by LTs and reduced by PGE₂ during bacterial pneumonia (14, 30, 31). Because we observed differences in the levels of these eicosanoids in the lungs of mice after bacterial challenge in vivo, we asked if LT and PGE₂ synthesis were altered in AMs from *l/l* mice in vitro. PGE₂ production in AMs stimulated for 1 h with heat-killed *K. pneumoniae* was ∼2-fold greater in cells from *l/l* mice (*p* = 0.0018) (Fig. 6A). In contrast, using the same stimulus, cysLT (*p* = 0.04) and LTB₄ [data not shown (*p* = 0.03)] production was decreased in AMs from *l/l* mice. Next, we assessed the expression of 5-LO, COX-1, COX-2, and mPGEs-1, enzymes known to play
essential roles in LT and PGE$_2$ synthesis in AMs, respectively. In comparison with cells from WT animals, 5-LO protein expression in AMs from $l/l$ mice was reduced by $\sim 40\%$ ($p = 0.008$). Although there were no differences in COX-1 ($p = 0.86$) or COX-2 ($p = 0.90$) (data not shown) expression, we did find a 90% increase in the levels of mPGE$_1$ in AMs from $l/l$ mice ($p = 0.04$). These changes in enzyme expression therefore explain the reduced LT and enhanced PGE$_2$ production in $l/l$ mice.

Elevated PGE$_2$ production mediates enhanced cAMP levels in AMs from $l/l$ mice

An important mechanism by which LTs and PGE$_2$ differentially regulate AM phagocytosis and killing of $K. pneumoniae$ in vitro is via decreases and increases, respectively, in the levels of the second messenger cAMP, which is known to inhibit phagocytosis and bacterial killing (30, 31). First, we confirmed that AMs from $l/l$ mice produced more PGE$_2$ than cells from WT mice ($p = 0.001$) after stimulation with heat-killed $K. pneumoniae$ and this response could be blocked with indomethacin (Fig. 6C). Next, we assessed intracellular cAMP levels and observed that AMs from $l/l$ mice stimulated with $K. pneumoniae$ for 1 h produced twice as much cAMP compared with AMs from WT animals ($p = 0.001$). The increase in intracellular cAMP was completely blocked when cells from $l/l$ mice were pretreated with indomethacin (Fig. 6D). AMs from $l/l$ mice pretreated with LTB$_4$ did not significantly affect cAMP production after stimulation with $K. pneumoniae$ ($p = 0.37$) (data not shown). These results suggest that the increased production of PGE$_2$ mediates the increased cAMP levels in AMs from $l/l$ mice.

Indomethacin restores phagocytosis and bacterial killing in AMs from $l/l$ mice

We next assessed the ability of addition or blockade of lipid mediators to restore AM effector functions in vitro. As shown in Fig. 7A and 7B, we again observed deficient bacterial phagocytosis ($p = 0.002$) and killing ($p = 0.002$) in AMs from $l/l$ mice.
Although we have previously reported that exogenous administration of LTB₄ or cysteinylation leukotrienes (cys-LTs) augments phagocytosis and killing in AMs from WT mice and rats (27, 32), these lipids failed to improve these endpoints in cells from l/l mice (data not shown). However, blocking of PGE₂ production with indomethacin restored defective antimicrobial responses in AMs from l/l mice. These results suggest that the defects in pulmonary host defense against K. pneumoniae in vivo were mainly due to the enhanced production of PGE₂ in cells from l/l mice. They also imply that the LepR mutation in l/l mice impairs responsiveness to LTs.

**Discussion**

In this study, we report the novel observation that l/l mice, which lack LepR→ERK1/2 activation via Tyr⁹⁸⁵, exhibit greater susceptibility to Gram-negative pneumonia. The defect in pulmonary host defense in l/l mice was associated with impaired AM phagocytosis and killing of bacteria in vitro. In addition, we also observed increased PGE₂ and reduced LTs after bacterial challenge in the lung in vivo and in AMs after culture with heat-killed bacteria in vitro. Phagocytosis and killing could be restored if AMs from l/l mice were pretreated with the cyclooxygenase inhibitor indomethacin, which normalized eicosanoid synthesis and intracellular cAMP levels. These results provide novel insights into the role of LepR-mediated signaling in the innate immune response against bacterial infection.

There are now a number of reports demonstrating that leptin or LepR deficiency disables host defense against bacterial infections (12–15, 19, 33–35). Unlike other models of leptin or LepR deficiency, the l/l mouse is neither obese nor hyperglycemic and thus expresses high levels of this receptor are likely to be influenced by expression of both these transcription factors, and the lack of STAT3 activation is not essential for host defense, as ablating this pathway in s/s mice, which possess a mutant LepRb, were obese and resistant to pneumococcal pneumonia in vivo, and this was associated with improved AMs antibacterial functions in vitro (18). Notably, these mice were protected by an enhanced ability to produce LTs, and this improved AM antibacterial functions. In the current study, we observed that ablation of the LepRb→Tyr⁹⁵⁸ signaling may have reduced their expression in l/l mice (22, 39, 40). The LTs play a protective role in Klebsiella pneumonia, as 5-LO knockout mice exhibited increased lethality and reduced pulmonary bacterial clearance (41). Furthermore, LT production was diminished in AMs from leptin-deficient (ob/ob) mice, and exogenous leptin restored LT synthesis and AM phagocytosis and killing of K. pneumoniae in vitro (12, 27, 32). However, the provision of exogenous LTs did not reduce cAMP levels or restore antibacterial responses in AMs from l/l mice, suggesting a defect in LT receptor responsiveness or signaling. Further evaluation of this possibility is a focus of future investigation but beyond the scope of the current report.

One novel observation in this report was the enhanced synthesis of the immunosuppressive eicosanoid PGE₂ in the lungs and in AMs of l/l mice postinfection.

This enhancement was the result of the increased expression of mPGES-1 as demonstrated by immunoblot analysis. The mechanism underlying this enhancement is unknown and beyond the scope of this report. PGE₂ suppresses bacterial phagocytosis, ROI generation, and bacterial killing in AMs, and these effects are mediated through the E-prostanoid 2 receptor, a G protein-coupled receptor that activates adenylyl cyclase and increases intracellular cAMP levels (30, 31). We also demonstrated elevated cAMP levels in AMs from l/l mice stimulated with bacteria in vitro, and this response could be blocked with the cyclooxygenase inhibitor indomethacin. This approach also normalized impaired phagocytosis and killing of K. pneumonia in AMs from l/l mice in vitro. Other reports have also demonstrated that elevated pulmonary PGE₂ synthesis suppresses host defense against bacterial pneumonia in vivo, and this defect can be rescued with indomethacin or through genetic ablation of the E-prostanoid 2 receptor (36, 37).

These results suggest that the defects in host defense in l/l mice were largely due to the overproduction of PGE₂ during bacterial pneumonia.

Another unexpected and novel finding in this report was the lower levels of LTs produced by l/l mice after pulmonary bacterial challenge in vivo and in AMs in vitro. This result was likely due to reduced 5-LO protein whose expression is regulated by transcription factors Sp1 and Egr1 (38). Leptin is known to enhance the expression of both these transcription factors, and the lack of LepRb→Tyr⁹⁸⁵ signaling may have reduced their expression in l/l mice (22, 39, 40). The LTs play a protective role in Klebsiella pneumonia, as 5-LO knockout mice exhibited increased lethality and reduced pulmonary bacterial clearance (41). Furthermore, LT production was diminished in AMs from leptin-deficient (ob/ob) mice, and exogenous leptin restored LT synthesis and AM phagocytosis and killing of K. pneumoniae in vitro (12, 27, 32). However, the provision of exogenous LTs did not reduce cAMP levels or restore antibacterial responses in AMs from l/l mice, suggesting a defect in LT receptor responsiveness or signaling. Further evaluation of this possibility is a focus of future investigation but beyond the scope of the current report.

Our data implicate dysregulated eicosanoid generation in AMs in the phenotype observed in l/l mice after K. pneumoniae challenge in vivo and in vitro. On the basis of p-STAT3 staining as a surrogate marker for the expression of the LepRb in the murine lung inflated with PBS containing leptin, we have shown that the LepRb is expressed primarily in AMs and to a much lesser extent in alveolar epithelial cells (18). Therefore, only those cells that express high levels of this receptor are likely to be influenced by the lack of LepRb→Tyr⁹⁵⁸ signaling. The primary sources of LTs...
in the lung during bacterial pneumonia are the resident AMs and PMNs known to express high levels of 5-LO. As a consequence, we observed reduced production of LTs at both time points after K. pneumoniae challenge in vivo and in AMs vitro. In contrast, the expression mpGEs-1 is not limited to AMs and would be present in alveolar epithelial cells that do not express high levels of the LepRb (18, 42). Consistent with this, we observed increased PGE_2 production in AMs stimulated in vitro and 4 h postinfection in the lungs of l/l mice in vivo. Under these circumstances, the AM is the major source of PGE_2. Twenty-four hours after Klebsiella challenge, the alveolar epithelial cells are the major producers of PGE_2 in vivo, and there were no differences in lung PGE_2 levels between WT and l/l mice. The impairment in pulmonary bacterial clearance in l/l mice in vivo was therefore most likely due to the elevated levels of PGE_2 produced by AMs. PGE_2, by increasing intracellular cAMP, is known to impair AM phagocytosis and killing of bacteria and to reduce ROI production, all of which are required for the elimination of K. pneumoniae (27, 30, 31).

The reduced number of monocyte/macrophages recovered from the lungs of l/l mice 24 h after Klebsiella challenge was not due to impairments in either chemokine (MCP-1) production or peripheral blood monocyte counts (data not shown), which did not differ from those of WT mice. It is also unlikely that the reduction of LTs in the lungs of l/l mice was responsible for lower monocyte/macrophage counts 24 h after K. pneumoniae challenge, as no differences in lung leukocytes counts were reported in 5-LO knockout mice after K. pneumoniae challenge (41). Although we did not assess cell viability, we speculate that the reduced monocyte/macrophage population in the lung of l/l mice 24 h after K. pneumoniae challenge may reflect increased apoptosis of these cells, as leptin is known to enhance the survival of human monocytes via an ERK1/2-dependent pathway (43). In support of this speculation, Guo et al. (17) reported increased cell death and disruption of the intestinal epithelium in l/l mice after infection with E. histolytica. In contrast to monocytes/macrophages, the increased numbers of PMNs in BAL fluid and the peripheral blood of l/l mice 24 h after K. pneumoniae challenge were likely due to the higher pulmonary bacterial burdens in these animals. On the basis of this result, it appears that the observed defect in ROI generation in PMNs (data not shown), rather than recruitment, may have contributed to the impairment in pulmonary bacterial clearance in l/l mice at this later time point. Finally, it is acknowledged that other bactericidal mechanisms may be dysfunctional in leukocytes from the l/l animals.

In summary, we report for the first time, to our knowledge, that LepRb→Tyf<sup>983</sup> intracellular signaling plays a critical role in the host response against Gram-negative pneumonia in vivo and in leukocyte antibacterial functions in vitro. At present, defects in human LepRb→ERK activation have not been identified. However, a leptin receptor mutation was associated with greater susceptibility to intestinal parasitic infections in humans (44). A greater understanding of the role of leptin receptor signaling in host defense against infection will facilitate the development of targeted therapeutic interventions for the prevention and treatment of bacterial pneumonia.

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Disclosures
The authors have no financial conflicts of interest.

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