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## Ablation of Leptin Receptor Mediated Extracellular Regulated Kinase Activation Impairs Host Defense against Gram-Negative Pneumonia

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### Abstract

The adipocyte-derived hormone, leptin, plays an important role in regulating 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/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 antibacterial functions in vitro. *l/l* mice exhibited increased mortality and impaired pulmonary bacterial clearance following intratracheal challenge with *Klebsiella pneumoniae*. The synthesis of cysteinyl-LTs was reduced and that of PGE<sub>2</sub> enhanced in alveolar macrophages in vitro and the lungs of *l/l* mice following infection with *K.pneumoniae* in vivo. We also observed reduced phagocytosis and killing of *K. pneumoniae* in AMs from *l/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/l* mice. Pharmacologic blockade of PGE<sub>2</sub> synthesis reduced cAMP levels and overcame the defective phagocytosis and killing of bacteria in AMs from *l/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.

## Keywords

leptin; macrophage; pneumonia; *K. pneumoniae*; eicosanoids

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## Introduction

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 following 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 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 H<sub>2</sub>O<sub>2</sub> 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 form of the leptin receptor (LepRb) via the janus kinase and signal transducer and activator of transcription (JAK/STAT) and mitogen activated protein (MAP) kinase signaling pathways. Upon binding to its ligand (Figure 1), the LepRb activates the constitutively associated JAK2 tyrosine kinase to induce tyrosine phosphorylation-dependent signaling via several divergent pathways. JAK2 mediates phosphorylation of Tyr<sup>1138</sup> which binds and mediates the phosphorylation-dependent activation of the latent transcription factor, STAT3. Following nuclear translocation, STAT3 activates transcription of suppressor of cytokine signaling (SOCS)-3, a protein that inhibits JAK2 and STAT3 signaling during prolonged stimulation of the LepRb (20). LepRb mediated phosphorylation of Tyr<sup>1077</sup> activates STAT5 signaling (21). Finally, phosphorylation of Tyr<sup>985</sup> recruits binding partners SH2-containing tyrosine phosphatase (SHP-2) and growth factor binding 2 (GRB2) which activate extracellular signal-regulated kinase 1 and 2 (ERK 1/2)(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 ERK 1/2 pathway via the LepRb due to a substitution mutation at Tyr<sup>985</sup> with L<sup>985</sup>. Although a recent report by Guo et al. 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 (17).

The role of LepRb-mediated (LepRb→) signaling events in the innate immune response against bacterial infections is complex and difficult to study in vivo. Not all leptin receptor mutations result in impaired immunity. For example, we recently reported that disruption of leptin receptor 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 LepRb→ Tyr<sup>985</sup> by comparing the responses of wild type (WT) with *l/l* mice in a murine model of bacterial pneumonia. We demonstrate for the first time that *l/l* mice exhibit increased susceptibility to gram-negative pneumonia and this pathway plays an essential role in the innate immune response against bacterial pneumonia.

## Materials and Methods

### Animals

Heterozygous C57Bl/6 (back-crossed for 8 generations or greater) *Lepr<sup>Tm2mgmj/+</sup>* mice were intercrossed in the University of Michigan Unit for Laboratory Animal Medicine (ULAM, Ann Arbor, MI) to generate age- and gender-matched male and female (*Lepr<sup>Tm2mgmj/Tm2mgmj</sup>* *l/l* and wild type (+/+;WT) (littermates) 8 to 14 weeks 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  $2 \times 10^6$  cells per ml, and allowed to adhere to tissue-culture plates for 1 h (37°C, 5% CO<sub>2</sub>) (24). After replacing the media with RPMI 1640 containing 10% fetal bovine serum (Life Technologies, Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin (Pen/Strep) (Invitrogen), the cells were cultured overnight. PMNs were obtained from mice by peritoneal lavage 5 h after an intraperitoneal injection of a 1% glycogen solution in PBS as previously described (25).

### Immunoblot analysis

AMs obtained from WT and *l/l* mice were plated at  $4 \times 10^6$  cells per well and cultured overnight in RPMI 1640 containing fetal bovine serum and Pen/Strep. On the following day, the cells were prepared for lysis or cultured with media alone or leptin (50 ng/ml) for 5, 15, or 30 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), were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to nitrocellulose membranes. Membranes were probed with the rabbit polyclonal antibodies against *phospho*-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)(1:2000), p44/42 MAPK (ERK1/2) (1:2,000), 5-lipoxygenase (5-LO) (1:250), GAPDH (1:1000) (Cell Signaling Technology, Danvers, MA), Cyclooxygenase-1(COX-1) (Bio-Rad, Hercules, CA) (1:200) and -2 (COX-2) (1:200), or microsomal prostaglandin E synthase-1 (mPGEs-1) (1:200) (Cayman Chemical, Ann Arbor, MI). Primary antibodies were detected using alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (titer 1:5,000) and visualized with the ECF detection system (Amersham Pharmacia Biotech, Piscataway, NJ). The densities of the luminescent bands were quantitated in appropriately exposed nitrocellulose by using Image Reader (FujiFilm). The density value of the

pERK1/2, 5-LO, COX-1, COX-2, and mPGEs-1 blots were divided by the density value of the ERK1/2 (pERK1/2) or GAPDH (5-LO, COX-1, COX-2, and mPGEs-1) blots, respectively, to normalize the relative band densities.

### **K. 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 ( $A_{600}$ ). *K. pneumoniae* was then pelleted by centrifugation (13,000 rpm for 3 min) 2×, 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- $\mu$ l inoculum containing  $5 \times 10^3$  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* colony forming units (CFUs)**

Following intratracheal inoculation with *K. pneumoniae*, mice were evaluated for survival daily for 7 days. 4 and 24 h following *K. pneumoniae* challenge, mice were euthanized by CO<sub>2</sub> asphyxiation, and lungs and spleen were harvested for CFU determinations as previously described (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**

4 and 24 h post-infection, lung leukocytes were obtained from mice by BAL following CO<sub>2</sub> asphyxiation and differential counts were performed on cells following staining with a modified Wright-Geimsa stain (American Scientific Products, McGraw Park, IL). Blood was collected by cardiac puncture for peripheral white blood cell counts using a Hemavet cell analyzer (Drew Scientific) operated by the University of Michigan Unit for Laboratory Animal Medicine Animal Diagnostic Laboratory.

### **Determination of cytokines, cysteinyl-leukotrienes (cysLTs), leukotriene B<sub>4</sub> (LTB<sub>4</sub>), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and leptin**

In a separate group of mice, lungs obtained from euthanized mice 4 and 24 h post-infection, were homogenized and cytokine (CXCL2 [MIP-2], CCL2 [MCP-1], IL-6, IL-10, IL-12, and TNF- $\alpha$ ) (R&D Duoset, R&D Systems, Minneapolis, MN) and cysLTs, LTB<sub>4</sub>, and PGE<sub>2</sub> (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, fluorescein isothiocyanate (FITC)-labeled *S. pneumoniae* (24). Briefly, AMs obtained by BAL were adhered and seeded in replicates of 8 to 384-well tissue culture plates with opaque sides and optically clear bottoms (Costar, Corning Inc. Life Sciences, Lowell, MA) and cultured overnight with RPMI 1640 with 1% Pen/Strep and 10% fetal calf serum (Invitrogen). On the following day, FITC-labeled *K. pneumoniae* were opsonized with 3% immune serum as previously described (27). AMs pretreated with RPMI media alone, with indomethacin (10  $\mu$ M) (Cayman Chemical, Ann Arbor, MI) for 30 min, or cysLTs (100 nM) and LTB<sub>4</sub> (1 nM) (alone or together) for 15 min were incubated with opsonized FITC-*K. pneumoniae* using an 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 SPECTRAMax GEMINI EM fluorometer 485ex/535em (Molecular Devices, Sunnyvale, CA). The phagocytic index was calculated as previously described in relative fluorescence units (RFU) (18, 24). Three separate experiments were conducted with 8 replicate wells for every experimental condition and the relative fluorescence units were normalized to the control condition (untreated AMs from WT animals) in each experiment.

### Bactericidal Assays

The survival of internalized *K. pneumoniae* within the AM was quantified using a tetrazolium dye reduction assay, as described previously (27). Briefly,  $2 \times 10^5$ /mL AMs, prepared as described previously, were adhered in quintuplicate in 96-well, half-area, tissue culture dishes (Corning, Inc., Lowell, MA). Following 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 minutes and were infected with a 0.1-mL suspension of opsonized *K. pneumoniae* ( $1 \times 10^7$  colony-forming units (CFU)/ml); multiplicity of infection (MOI, 50:1) for 30 minutes to allow phagocytosis to occur. The AMs were then washed 3x 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). Based on 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 =  $100\% \times A_{595} \text{ control plate} / A_{595} \text{ experimental plate}$ .

### Reactive oxygen intermediate (ROI) production

AMs were adhered to 384-well plates at a concentration of  $1.25 \times 10^5$  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 H<sub>2</sub>DCF 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* (*K.p*) opsonized with 3% specific immune serum using a multiplicity of infection 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, Sunnyvale, CA) with excitation/emission setting at 493/522 nm.

### Assessment of Nitric oxide (NO) production

AMs were adhered to 96-well plates at a concentration of  $2 \times 10^5$  cells/well and cultured with DMEM supplemented with 1% sodium pyruvate (Invitrogen) containing 10% FCS and penicillin/streptomycin with or without 10 ng/ml lipopolysaccharide (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<sub>2</sub><sup>-</sup>) concentrations using a modified Griess reaction with a commercially available assay kit, according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

### Measurement of intracellular cyclic adenosine monophosphate (cAMP) by AMs in vitro

AMs were cultured overnight in 96-well plates in RPMI 1640 with 10% fetal calf serum and 1% Pen/Strep at concentrations of  $2 \times 10^6$  cells/well. On the following day, the cell culture media was replaced with warm RPMI and AMs were incubated for 30 min in the presence or absence of indomethacin (10 µM) with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (EMD Biosciences) for 30 min prior to stimulation with *K.p*.

opsonized with 3% rat specific immune serum using a multiplicity of infection 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 enzyme-linked immunosorbent assay 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 analysis of variance (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  $\pm$  standard error of the mean unless otherwise noted.

## Results

### Substitution of LepRb Tyr<sup>985</sup> with L<sup>985</sup> in *l/l* mice abrogates LepRb mediated ERK1/2 activation

In order to confirm that *l/l* mice lack the ability to signal via LepRb Tyr<sup>985</sup>, we assessed ERK 1/2 activation using immunoblot analysis of pERK 1/2 in AMs obtained from WT and *l/l* mice cultured with leptin. As shown in Figure 1C, levels of total ERK 1/2 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 pERK1/2 as determined by a 50% increase in pERK1/2 ( $p=0.0002$ ). We conducted time course experiments for ERK activation (pERK) (i.e. 5, 15, and 30 mins after stimulation with leptin) and only the blots from cells stimulated for 30 min are shown since this represents the peak of this response. In contrast, we did not observe any increases in pERK 1/2 levels in AMs from *l/l* mice following 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 $\rightarrow$ STAT3 or STAT5 are normal as previously reported (21, 23). In addition, hypothalamic pERK activation was not observed in a previous report using *l/l* mice treated with much higher doses of leptin (5  $\mu$ g/g of body wt) (23). Blood leptin levels were slightly lower ( $p=0.04$ ) in the *l/l* mice ( $2.9 \pm 0.5$  ng/ml) compared with that of WT animals ( $4.5 \pm 0.9$  ng/ml) as previously reported (23). These data indicate that leptin induces phosphorylation of ERK 1/2 via the LepRb Tyr<sup>985</sup> and that this pathway is abrogated in AMs from *l/l* mice.

### *l/l* mice exhibit greater mortality and reduced pulmonary bacterial clearance following *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). In order to determine if intracellular signals arising from the LepRb Tyr<sup>985</sup> play a role in pulmonary host defense against gram-negative pneumonia, we compared the responses of WT and *l/l* mice following an intratracheal challenge with *K. pneumoniae*. As shown in Figure 2A, *l/l* mice exhibited substantially lower survival (27%) as compared with WT (65%,  $p=0.04$ ) following *K. pneumoniae* challenge 7 days post-infection. Since 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 post-infection. We chose these time points since we observed that the first death recorded for an *l/l* mouse occurred 48 h after *K. pneumoniae* challenge. As shown in Figure 2B, bacterial burdens were approximately 1-log fold greater after 4 h ( $p=0.02$ ) and 4-log fold higher at 24 h ( $p=0.0001$ ) in *l/l* as 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 ( $p=0.73$ ) post-infection.

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 following *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). In order to determine if the ablation of LepRb Tyr<sup>985</sup> signaling alters leukocyte recruitment to the lung during bacterial pneumonia, we recovered leukocytes by lavage in mice 4 and 24 h post-infection since these were the time points when we observed differences in pulmonary bacterial burdens. As shown in Figure 3, there were no differences ( $p=0.29$ ) between WT and *l/l* mice in total or differential leukocyte counts in BALF 4 h after *K. pneumoniae* challenge. However, we did find lower BALF 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*:  $6.7 \times 10^6$  vs. WT:  $4.1 \times 10^6$  WBC/ml) ( $p=0.04$ ) and elevated PMN counts (*l/l*:  $3.8 \times 10^6$  vs. WT:  $1.9 \times 10^6$ ) ( $p=0.04$ ) in the *l/l* mice 24 h post-infection. 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 post-*K. pneumoniae* challenge

In order 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 cysteinyl-LTs, PGE<sub>2</sub>, and cytokine levels after bacterial challenge. As shown in Figure 4A, there were no differences in lung homogenate cytokines known to play an important role in antibacterial host defense (IL-6, TNF- $\alpha$ , IL-10, IL-12, MIP-2, TNF- $\alpha$ ) 4 h post-infection. However, we did find major differences in lipid mediators. COX derived PGE<sub>2</sub> was elevated ( $p=0.02$ ) while the 5-LO derived cysteinyl-LTs were reduced ( $p=0.01$ ) in *l/l* mice at this time point. Reduced cysLT levels persisted 24 h post-infection in the lungs of *l/l* mice ( $p=0.02$ ) (Figure 4C) while no differences in PGE<sub>2</sub> or cytokines were observed ( $p=0.93$ ) (Figure 4D).

### Impaired phagocytosis, killing and reactive oxygen intermediate production in AMs from *l/l* mice

The observed 1-log fold elevation of bacterial CFUs in *l/l* mice 4 h post-infection 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 Figure 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 Figure 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 vs *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, as compared with that of WT animals, by approximately 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 nitric oxide production following stimulation overnight with LPS and IFN- $\gamma$  ( $p=0.15$ ) (data not shown). These results indicate that the LepRb $\rightarrow$ Tyr<sup>985</sup> 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.

### **Diminished LTs and enhanced PGE<sub>2</sub> 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 PGE<sub>2</sub> during bacterial pneumonia (14, 30–31). Since we observed differences in the levels of these eicosanoids in the lungs of mice after bacterial challenge in vivo, we asked if LT and PGE<sub>2</sub> synthesis were altered in AMs from *l/l* mice in vitro. PGE<sub>2</sub> production in AMs stimulated for 1 h with heat killed *K. pneumoniae* was approximately two-fold greater in cells from *l/l* mice ( $p=0.0018$ ) (Figure 6A). In contrast, using the same stimulus, cysLT ( $p=0.04$ ) and LTB<sub>4</sub> (not shown ( $p=0.03$ )) production were 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<sub>2</sub> synthesis in AMs, respectively. In comparison with cells from WT animals, 5-LO protein expression in AMs from *l/l* mice was reduced by approximately 40% ( $p=0.008$ ). While 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 mPGEs-1 in AMs from *l/l* mice ( $p=0.04$ ). These changes in enzyme expression therefore explain the reduced LT and enhanced PGE<sub>2</sub> production in *l/l* mice.

### **Elevated PGE<sub>2</sub> production mediates enhanced cAMP levels in AMs from *l/l* mice**

An important mechanism by which LTs and PGE<sub>2</sub> 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<sub>2</sub> than cells from WT mice ( $p=0.001$ ) following stimulation with heat killed *K. pneumoniae* and this response could be blocked with indomethacin (Figure 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 (Figure 6D). AMs from *l/l* mice pretreated with LTB<sub>4</sub> did not significantly affect cAMP production following stimulation with *K. pneumoniae* ( $p=0.37$ ) (data not shown). These results suggest that the increased production of PGE<sub>2</sub> 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 Figure 7A and B, we again observed deficient bacterial phagocytosis ( $p=0.002$ ) and killing ( $p=0.002$ ) in AMs from *l/l* mice. While we have previously reported that exogenous administration of LTB<sub>4</sub> or cysLTs 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 PGE<sub>2</sub> 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 PGE<sub>2</sub> 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 LepRb  $\rightarrow$  ERK1/2 activation via Tyr<sup>985</sup> 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 PGE<sub>2</sub> and reduced LTs after bacterial challenge in the lung in vivo and in AMs following 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 leptin receptor mediated signaling in the innate immune response against bacterial infection.

There are now a number of reports demonstrating that leptin or leptin receptor deficiency disables host defense against bacterial infections (12–15, 19, 33–35). Unlike other models of leptin or leptin receptor deficiency, the *l/l* mouse is neither obese nor hyperglycemic and thus provides an excellent model for assessing the importance of distinct LepR mediated signaling events in host defense against bacterial infection in the absence of endocrine abnormalities. We recently reported that LepRb–STAT3 activation is not essential for host defense, since 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). Interestingly, 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 $\rightarrow$ Tyr<sup>985</sup> pathway in *l/l* mice, which were lean, resulted in defective host defense against *K. pneumoniae* in vivo and diminished AM effector functions in vitro. These differences were also most likely due to alterations in eicosanoid production.

One novel observation in this report was the enhanced synthesis of the immunosuppressive eicosanoid, PGE<sub>2</sub>, in the lungs and in AMs of *l/l* mice after infection. 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 this scope of this report. PGE<sub>2</sub> suppresses bacterial phagocytosis, ROI generation, and bacterial killing in AMs and these effects are mediated through the E-prostanoid 2 receptor (EP2), a G $\alpha_s$ -protein coupled receptor which activates adenylate 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. pneumoniae* in AMs from *l/l* mice in vitro. Other reports have also demonstrated that elevated pulmonary PGE<sub>2</sub> synthesis suppresses host defense against bacterial pneumonia in vivo and this defect can be rescued with indomethacin or through genetic ablation of the EP2 receptor (36–37). These results suggest that the defects in host defense in *l/l* mice were largely due to the overproduction of PGE<sub>2</sub> during bacterial pneumonia.

Another unexpected and novel finding in this report was the lower levels of LTs produced by *l/l* mice following 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 $\rightarrow$ Tyr<sup>985</sup> signaling may have reduced their expression in *l/l* mice (22, 39–40). The LTs play a protective role in *Klebsiella pneumoniae* since 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 following *K. pneumoniae* challenge in vivo and in vitro. Based on pSTAT3 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→Tyr<sup>985</sup> 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 following *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 which do not express high levels of the LepRb (18, 42). Consistent with this, we observed increased PGE<sub>2</sub> production in AMs stimulated in vitro and 4 h post-infection in the lungs of *l/l* mice in vivo. Under these circumstances, the AM is the major source of PGE<sub>2</sub>. 24 h post-Klebsiella challenge, the alveolar epithelial cells are the major producers of PGE<sub>2</sub> in vivo and there were no differences in lung PGE<sub>2</sub> 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<sub>2</sub> produced by AMs. PGE<sub>2</sub>, 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 24h post-*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 that 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 post-*K. pneumoniae* challenge since no differences in lung leukocytes counts were reported in 5-LO knockout mice following *K. pneumoniae* challenge (41). While we did not assess cell viability, we speculate that the reduced monocyte/macrophage population in the lung of *l/l* mice 24 h post-*K. pneumoniae* challenge may reflect increased apoptosis of these cells since leptin is known to enhance the survival of human monocytes via a ERK1/2 dependent pathway (43). In support of this speculation, Guo et al. reported increased cell death and disruption of the intestinal epithelium in *l/l* mice following infection with *E. histolytica* (17). In contrast to monocytes/macrophages, the increased numbers of PMNs in BALF 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. Based on 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, that LepRb→Tyr<sup>985</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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## Non-standard abbreviations

<b>AM</b>	alveolar macrophage
<b>BAL</b>	bronchoalveolar lavage
<b>COX-1</b>	cyclooxygenase-1
<b>cysLTs</b>	cysteinyl-leukotrienes
<b>ERK</b>	extracellular regulated kinase
<b>GRB2</b>	growth factor binding 2
<b>H.K. <i>K.p.</i></b>	heat-killed <i>K.pneumoniae</i>
<b>JAK</b>	janus kinase
<b>5-LO</b>	5-lipoxygenase
<b>LepRb</b>	long isoform of the leptin receptor
<b>LT</b>	leukotriene
<b>LTB<sub>4</sub></b>	leukotriene B <sub>4</sub>
<b>mPGEs-1</b>	microsomal prostaglandin E synthase-1
<b>MOI</b>	multiplicity of infection
<b>PGE<sub>2</sub></b>	prostaglandin E <sub>2</sub>
<b>STAT3</b>	signal transducer and activator of transcription 3
<b>SOCS3</b>	suppressor of cytokine signaling 3

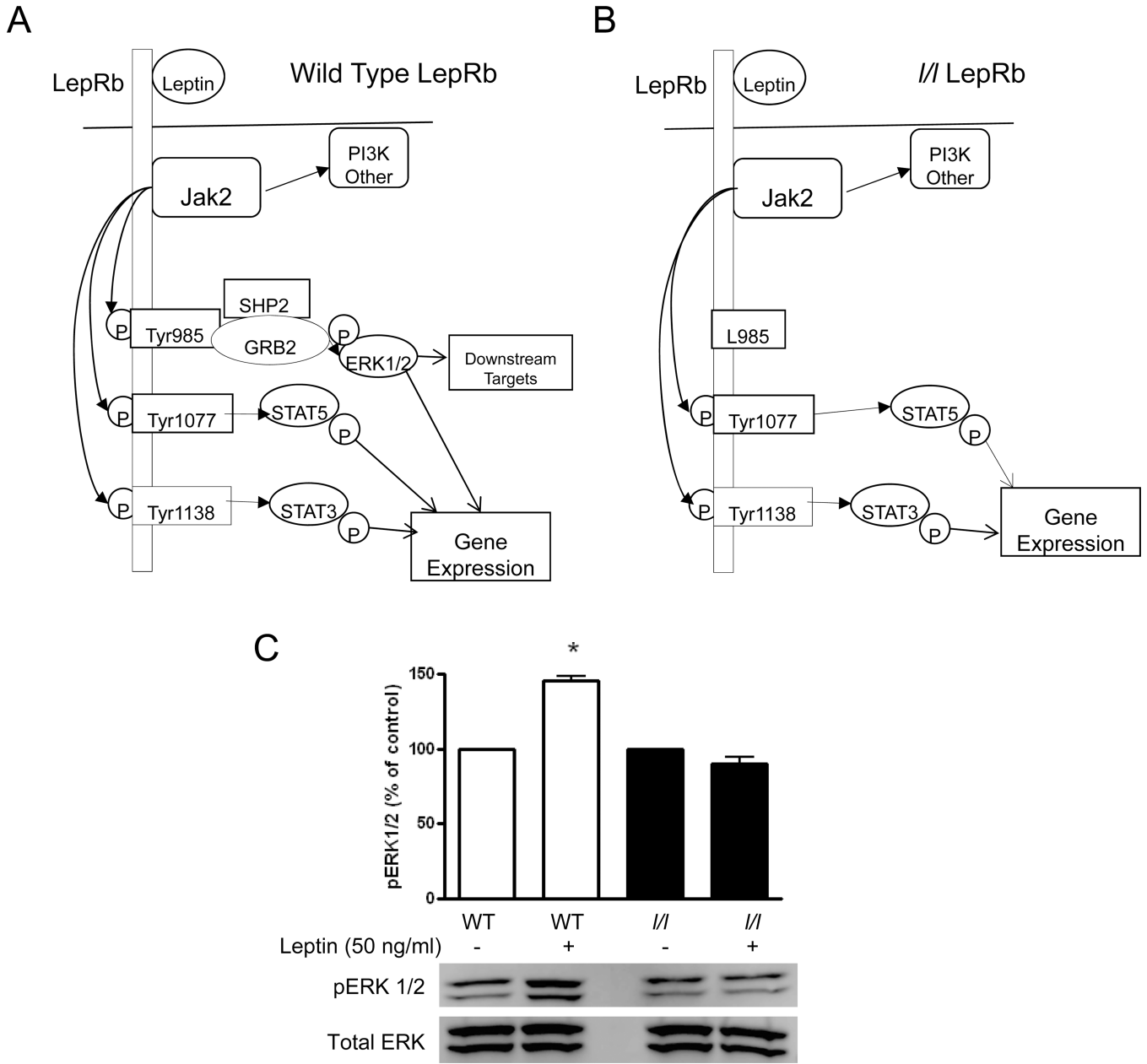
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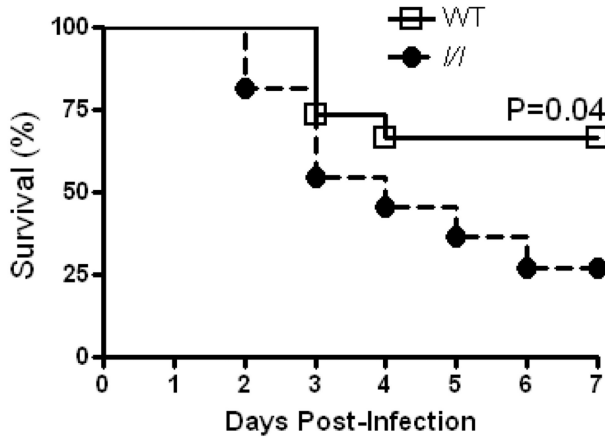


**Figure 1.** LepRb signaling events. A. In wild type (WT) mice (A), leptin binding activates the LepRb-associated janus associated kinase 2 (Jak2), a tyrosine kinase that mediates tyrosine phosphorylation of LepRb to promote several intracellular signaling events: 1) LepRb Tyr<sup>985</sup> recruits SH2-containing tyrosine phosphatase (SHP-2) and growth factor binding 2 (GRB2) and to promote the activation of extracellular regulated kinases 1 and 2 (ERK1/2). ERK1/2 phosphorylates downstream targets and induces the transcription of genes. 2) Phosphorylated Tyr1107 binds and mediates the phosphorylation-dependent activation of STAT5 and 3) Tyr1138 recruits STAT3, which promotes the transcription many different genes. In // mice (B), substitution of LepRb Tyr<sup>985</sup> with LepRb L<sup>985</sup> prevents leptin mediated ERK 1/2 activation in AMs from // mice. AMs obtained from WT and // mice were cultured with media alone or with exogenous leptin (50 ng/ml) for 30 min and

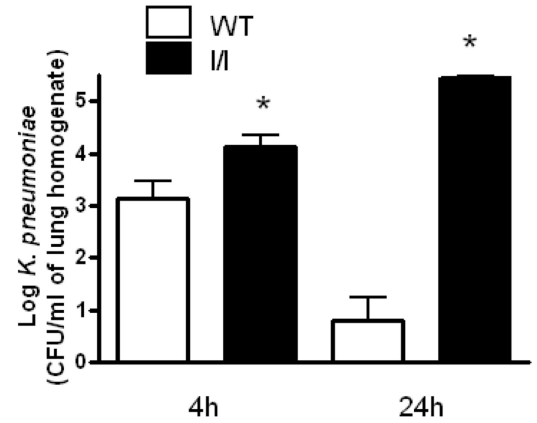
evaluated for pERK1/2 and total ERK 1/2 by immunoblot analysis. Protein levels were determined by densitometric analysis of immunoblots from 5 separate experiments (B).\*,  $P < 0.001$  vs untreated WT using the students  $t$ -test.



A



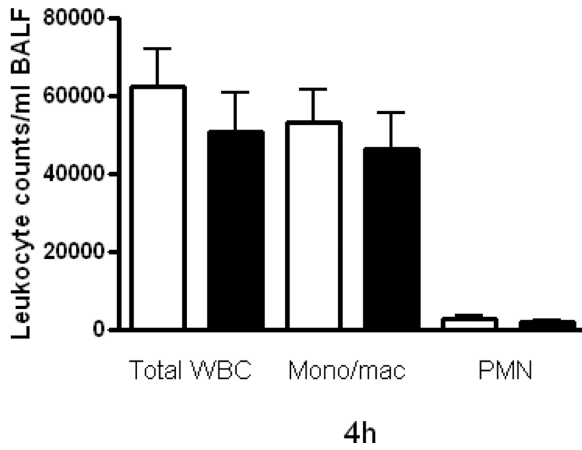
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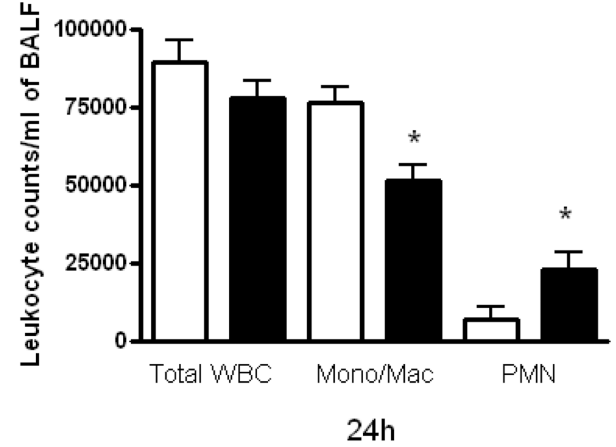
**Figure 2.**

Reduced survival and impaired pulmonary bacterial clearance in *I/I* mice following an intratracheal challenge with *K. pneumoniae*. Wild type (WT) and *I/I* mice were challenged with  $5 \times 10^3$  CFU of *K. pneumoniae* via the intratracheal route and monitored for survival for 7 days (A). In another group of animals, WT (open bars) and *I/I* (solid bars) mice were euthanized 4 and 24 h after infection and lungs were harvested and bacterial burdens in tissue homogenates were determined by counting colony forming units (CFU) as described in *Materials and Methods* (B). \* $P < 0.05$  compared to WT using a log-rank test. Survival curves represent an n=11–15 mice per group from 3 separate experiments. \* $P < 0.05$  compared to WT levels at 4 and 24h after infection using a *Student's t-test*. Bars represent the mean  $\pm$  SEM of n=10 mice per group from three separate experiments.

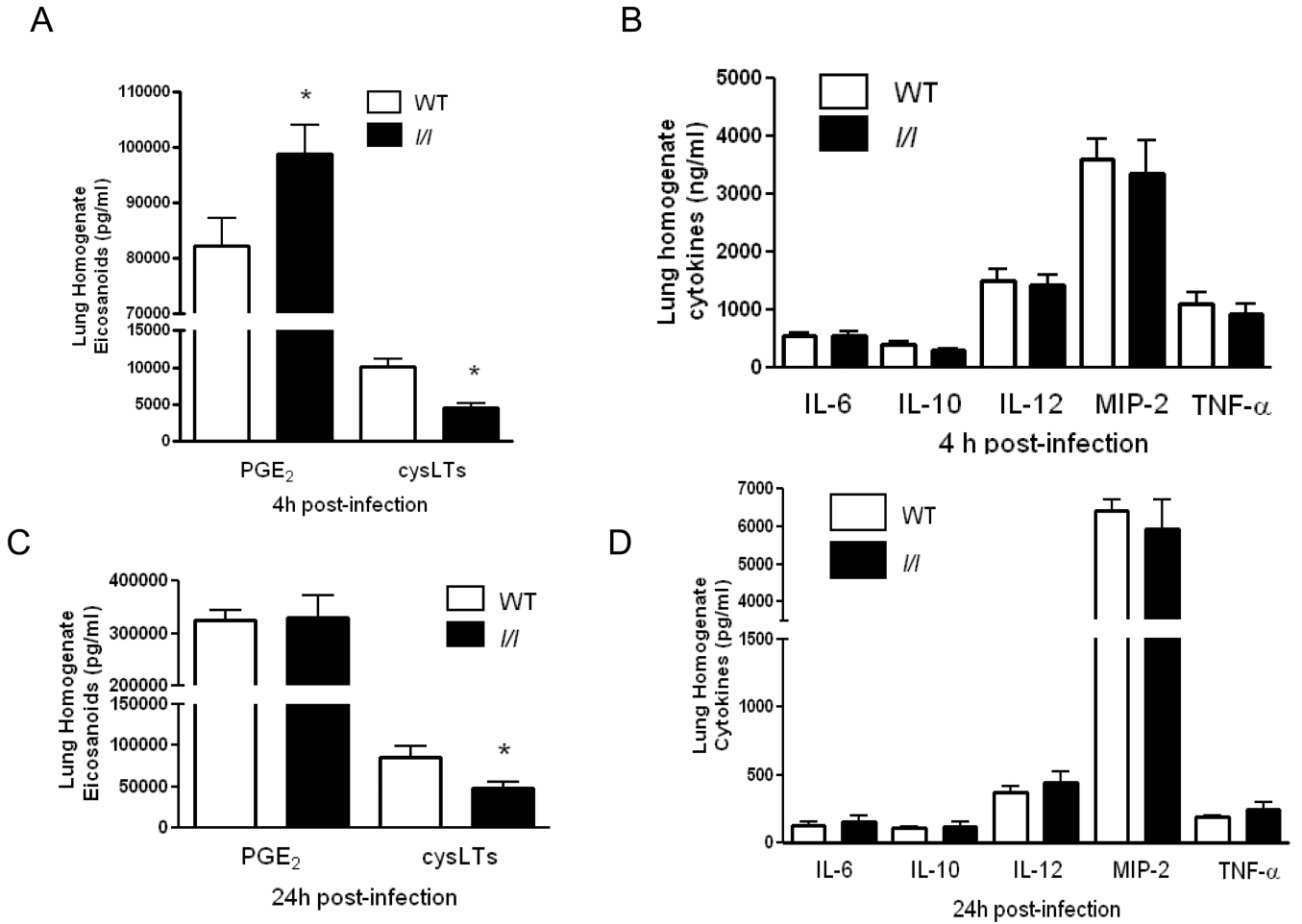
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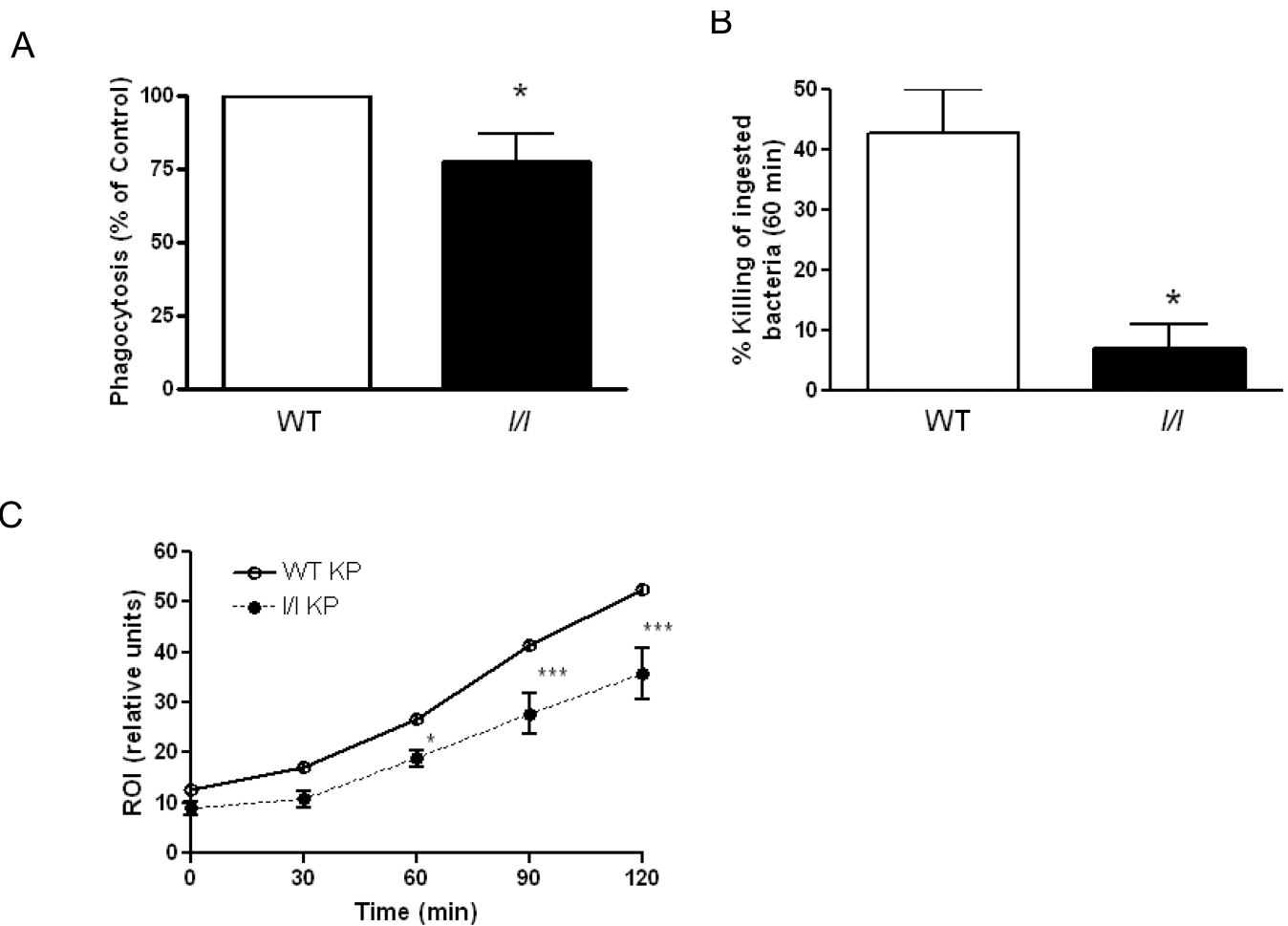
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**Figure 3.**

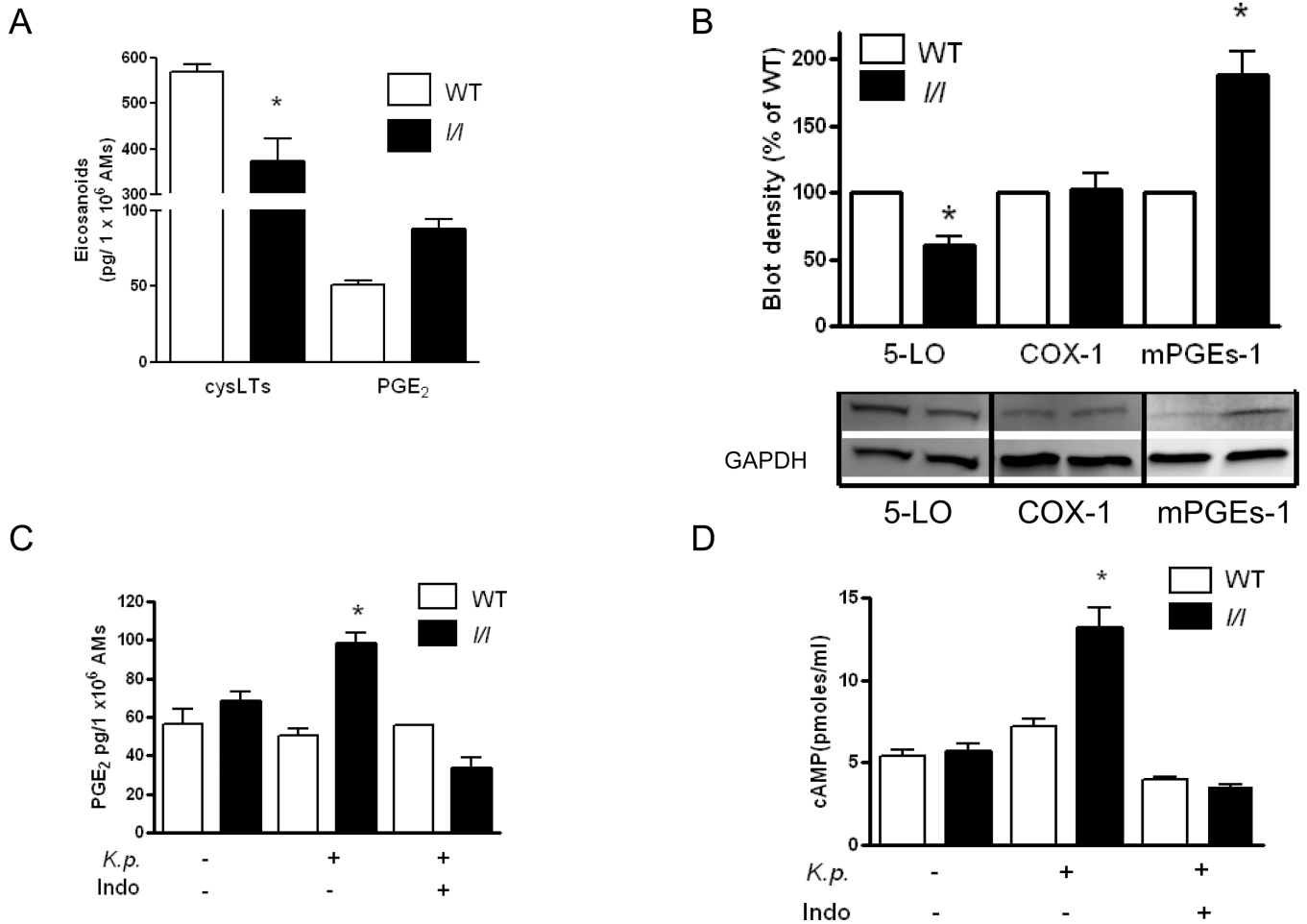
Leukocyte recruitment in wild type (WT) and *I/I* mice following intratracheal *K. pneumoniae* infection. WT (open bars) and *I/I* (solid bars) mice were infected via the intratracheal route with  $5 \times 10^3$  CFUs of *K. pneumoniae*. 4 and 24 h later, cells were recovered by bronchoalveolar lavage 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  $\pm$  SEM of n=10 mice per group. \* $P < 0.05$  compared to WT using a *Student's t-test*.



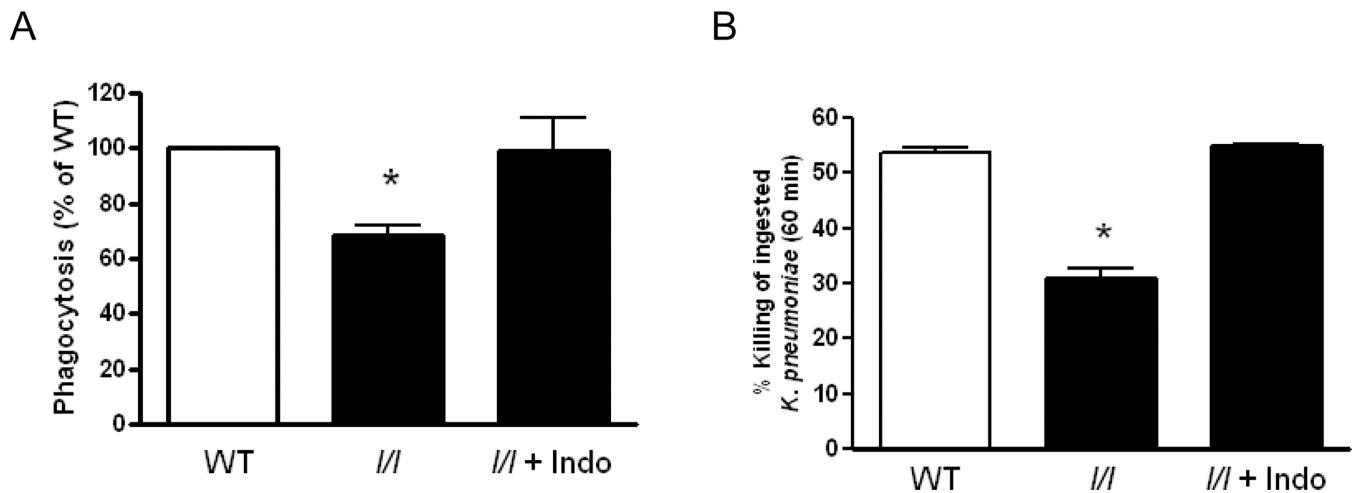
**Figure 4.** Elevated prostaglandin E<sub>2</sub> and reduced cysteinyl-leukotrienes (cysLT) in // mice in lung homogenates following intratracheal *K.pneumoniae* challenge. Wild type (WT) (open bars) and // (solid bars) mice were infected via the intratracheal (i.t.) route with *K.pneumoniae*. Lung homogenates were prepared from mice 4 (A and B) and 24h (C and D) after i.t. bacterial challenge and eicosanoids, cysteinyl-leukotrienes (cysLTs) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and cytokines (IL-6, IL-10, IL-12, MIP-2, and TNF- $\alpha$ ) were determined as mentioned in *Materials and Methods*. \**P* < 0.05 compared to WT levels at 4 and 24 hr after infection using a *Student's t-test*. Bars represent the mean  $\pm$  SEM of n=5–10 mice per group.



**Figure 5.** Impaired phagocytosis, bacterial killing, and reactive oxygen intermediate production in AMs from *I/I* mice. AMs obtained from WT (open bars) and *I/I* (solid bars) mice were assessed for their ability to phagocytose (A) and kill ingested *K. pneumoniae* opsonized with immune serum (B) according to the procedures described in the *Materials and Methods* section. \* $P < 0.05$  compared to WT using a student *t*-test. Bars represent the mean  $\pm$  SEM of at least 3 separate experiments. AMs from WT (open circles) and *I/I* (closed circles) mice were cultured with H2DCF for 1 h, and then stimulated with heat-killed *K. pneumoniae* opsonized with immune serum using a multiplicity of infection of 100:1 to assess reactive oxygen intermediate production (ROI) (C). ROI production was assessed using a fluorometer and expressed as relative fluorescence units. The data represent the mean of three experiments completed in quadruplicate for each time point using a two way analysis of variance. \* $P < 0.05$  and \*\*\* $P < 0.001$  compared WT.

**Figure 6.**

Eicosanoid synthesis, eicosanoid biosynthetic enzyme expression, and cAMP levels in AMs from WT (open bars) and *I/I* (solid bars) mice. AMs from WT (open bars) and *I/I* (solid bars) mice were stimulated with heat killed *K. pneumoniae* (*K.p.*) opsonized with specific immune serum for 1 h and cysteinyl-leukotrienes (cysLT) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) were determined by ELISA as described in the *Materials and Methods* section (A). Immunoblots for 5-lipoxygenase (5-LO), cyclooxygenase-1 (COX-1), microsomal prostaglandin E synthase-1 (mPGEs-1), and glyceraldehydes-phosphate-dehydrogenase (GAPDH) (protein loading control) in AM lysates from a single experiment that is representative of a total of 3 independent experiments. Bars represent the mean arbitrary densitometric units  $\pm$  SEM of immunoblots from 3 separate experiments (B). AMs cultured with and without indomethacin (Indo) (10  $\mu$ M) for 30 min were stimulated with or without heat killed *K. pneumoniae* (*K.p.*) opsonized with immune serum for 1 h and the cell culture media was assayed for PGE<sub>2</sub> (C) and AM lysates were assayed for cAMP as described in the *Materials and Methods* section (D). \*  $P < 0.05$  vs WT using the students *t*-test.



**Figure 7.**

Inhibition of PGE<sub>2</sub> synthesis with indomethacin restores bacterial phagocytosis and killing in AMs from *l/l* mice. AMs obtained from WT and *l/l* mice were cultured with and without indomethacin (Indo) (10 μM) for 30 min prior to the addition of *K. pneumoniae* opsonized with immune serum. Phagocytosis (A) and bacterial killing of *K. pneumoniae* ingested by AMs from WT and *l/l* mice were assessed according to procedures mentioned in the *Materials and Methods* section. Bars represent the mean ± SEM of at least 3 separate experiments. \*P<0.01 vs WT and *l/l* + Indo by ANOVA using the Bonferroni test for mean separation.