ORIGINAL RESEARCH

A Comparative Study of Lung Host Defense in Murine Obesity Models
Insights into Neutrophil Function

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Abstract

We have shown that obesity-associated attenuation of murine acute lung injury is driven, in part, by blunted neutrophil chemotaxis, yet differences were noted between the two models of obesity studied. We hypothesized that obesity-associated impairment of multiple neutrophil functions contributes to increased risk for respiratory infection but that such impairments may vary between murine models of obesity. We examined the most commonly used murine obesity models (diet-induced obesity, db/db, CPE$^{fat/fat}$, and ob/ob) using a *Klebsiella pneumoniae* pneumonia model and LPS-induced pneumonitis. Marrow-derived neutrophils from uninjured lean and obese mice were examined for *in vitro* functional responses. All obesity models showed impaired clearance of *K. pneumoniae*, but in differing temporal patterns. Failure to contain infection in obese mice was seen in the db/db model at both 24 and 48 hours, yet this defect was only evident at 24 hours in CPE$^{fat/fat}$ and ob/ob models, and at 48 hours in diet-induced obesity. LPS-induced airspace neutrophilia was decreased in all models, and associated with blood neutropenia in the ob/ob model but with leukocytosis in the others. Obese mouse neutrophils from all models demonstrated impaired chemotaxis, whereas neutrophil granulocyte colony-stimulating factor–mediated survival, LPS-induced cytokine transcription, and mitogen-activated protein kinase and signal transducer and activator of transcription 3 activation in response to LPS and granulocyte colony-stimulating factor, respectively, were variably impaired across the four models. Obesity-associated impairment of host response to lung infection is characterized by defects in neutrophil recruitment and survival. However, critical differences exist between commonly used mouse models of obesity and may reflect variable penetrance of elements of the metabolic syndrome, as well as other factors.

Keywords: obesity; acute respiratory distress syndrome; pneumonia; innate immunity; neutrophil

Clinical Relevance

A variety of obese animal models are used to study the effects of obesity on pulmonary host defense. This study demonstrates that obesity is associated with impaired pulmonary host defense. However, the course and severity of infection, as well as the inflammatory response, may vary depending on the obesity model examined, possibly related to variable expression of the metabolic syndrome in these models.

Obesity is a rapidly expanding global epidemic, with a rapidly rising prevalence of overweight and obese body mass index in both adults and children, which have increased by 27.5 and 47.1%, respectively, in the past 30 years (1). Recent studies have demonstrated that human obesity is associated with increased risk for developing bacterial and viral respiratory infections (2), as well as greater incidence of acute respiratory distress syndrome (ARDS) (3). Interestingly, however, we

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and others have shown that, once ARDS has developed, obesity is also associated with rapid attenuation of the inflammatory response and improved outcomes in both patients (3, 4) and animal models (5). Recently, using both diet-induced obese (DIO) and hyperphagic mutant obese (db/db) mice, we showed impaired pulmonary innate immune responses after LPS-induced lung injury, as well as defects in neutrophil chemoattractant response in the obese mice, suggesting that obesity-associated neutrophil dysfunction may, in part, underlie the attenuated inflammatory response seen in these animals (5). However, significant differences in the degree of impairment were noted between the two models of obesity studied, despite being of similar weights. Others studying the effects of obesity on hyperoxic and ozone-induced lung injury have shown that obese mice (db/db and ob/ob) have reduced lung injury and mortality, but to varying degrees (6–8).

As a corollary to the impaired inflammatory response seen in obese ARDS models, we and others have shown that pulmonary host defense is impaired after both influenza (9, 10) and bacterial pneumonias (11, 12), with consequent lung injury and death. Recent clinical studies have demonstrated associations between obesity and risk for both bacterial and viral pneumonias, as well as increased disease severity and mortality (13–15), whereas others have suggested a protective effect of increasing body mass index on mortality in this setting (16, 17). Similar to these human studies, variable results were also found using mouse models of obesity. Susceptibility to and outcome of bacterial respiratory infection was increased in obese ob/ob mice compared with their lean controls (11, 18), whereas no differences were observed between obese CPE$^{fat/fat}$ mice and their lean controls (19). Reasons for this inconsistency may include pathogen-specific defects in the immune response or differences in the metabolic state (lipid, glucose, and adipokine levels, among others) of individual subjects with obesity. Differences between these and other mouse models of obesity may, in part, determine the effects on the pulmonary immune response and, subsequently, the susceptibility to and outcome of respiratory infections. However, there are limited data available comparing different murine models of obesity and their metabolic state on the level of immune cell dysfunction and, consequently, the effects on host defense.

A variety of mouse models have been used to study obesity. Although it has been suggested that human obesity may be best mimicked by the DIO mouse model of high-fat diet (20–22), three hyperphagic mutant models are also commonly reported, including leptin "resistant" db/db mice (long form of leptin receptor (*ObRb*) deficient) (23), aleptinemic ob/ob mice (leptin [*ob*] deficient) (24), and hyperphagic CPE$^{fat/fat}$ mice (carboxypeptidase-E deficient) (25). Studies in these models have suggested that obesity-associated defects may exist in both the innate and adaptive immune responses (5, 7, 9–11, 18, 19, 26–32). However, inconsistencies exist in the published literature, suggesting that these models may display different features/hallmarks of the obese state or the underlying defect leading to obesity. Therefore, important caveats may exist when studying the available obese mouse models, which should be taken into account when extrapolating to human obesity.

In the current study, we examine obesity-associated defects in the innate immune response to bacterial pneumonia, and implicate elements of neutrophil impairment as an underlying mechanism. Furthermore, we detail important differences between commonly used mouse models of obesity related to respiratory infection and the underlying role of neutrophil dysfunction. We present evidence of a consistent failure to contain bacterial growth after respiratory infection in obese mice. However, the temporal course and severity of the infection, as well as the underlying mechanisms, appear to vary significantly with the mouse model used. Furthermore, we show that obesity is associated with numerous defects in neutrophil function, including impaired chemotaxis, cytokine transcription, and cell survival, and link these to obesity-associated defects in intracellular signaling responses.

Materials and Methods

Mice

For the DIO model, C57BL/6J mice (Harlan, Indianapolis, IN) were fed high-fat (60% fat) versus normal-fat (10% fat) chow (Research Diets, New Brunswick, NJ) for 20 weeks. In genetic models of obesity, homozygous B6.BKS(D)-LepR$^{db/J}$ (*db/db*) mice (lacking ObRb, the long form of the leptin receptor), homozygous B6.HRS (BKS)-CPE$^{fat/J}$ (CPE$^{fat/fat}$) mice (hyperphagic due to a mutation in the carboxypeptidase-E gene), homozygous B6.Cg-lep$^{ob/J}$ (*ob/ob*) mice (leptin deficient) (on a C57BL/6 background; obtained from Jackson Labs, Bar Harbor, ME), and their lean heterozygous littermates were examined at 12–16 weeks (genetic models of obesity) or 26–30 weeks (DIO) of age. Mice were considered "obese" once the difference between their body weight and that of controls reached at least 20 g (33). Mutant obese mice and their littermate controls were housed in the same cages. Experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by the Institutional Animal Care and Use Committee of the University of Vermont (Burlington, VT).

Murine Exposures

Klebsiella pneumoniae infections were induced by oropharyngeal aspiration of *K. pneumoniae* (43,816 serotype 2, 2×10^3 CFU; ATCC, Manassas, VA) after brief anesthesia with isofluorane (34). LPS-induced pneumonitis was induced by exposure to nebulized LPS (*Escherichia coli* 0111:B4 LPS; Sigma, St. Louis, MO) (5).

Preparation of Morphologically Mature Murine Bone Marrow Neutrophils

Mature bone marrow neutrophils were isolated using a three-step Percoll gradient, as previously described (35).

Neutrophil Chemotaxis

Chemotaxis of bone marrow–derived neutrophils was assayed in response to keratinocyte chemoattractant (KC) (R&D systems, Minneapolis, MN) and *N*-formylmethionyl-leucyl-phenylalanine (fMLP) (Sigma) using a 48-well modified Boyden chamber with 5-μm pore polycarbonate membranes (both NeuroProbe Inc., Gaithersburg, MD), as previously described (12, 36).

Neutrophil Apoptosis

Bone marrow–derived neutrophils were incubated for 6 hours with 200 ng/ml

of Fas-ligand (FasL; ENZO Life Sciences, Farmingdale, NY) and with or without 25 ng/ml recombinant granulocyte colony stimulating factor (rG-CSF) (Amgen, Thousand Oaks, CA), or with 25 ng/ml rG-CSF alone. Cells were counted using a hemocytometer (Sigma, St. Louis, MO), and trypan blue was used for dead cell exclusion.

Neutrophil Cytokine Transcription

Bone marrow–derived neutrophils were stimulated with LPS (100 ng/ml) for 4 hours at 37°C, and IL-6, IL-1β, KC, TNF-α, macrophage inflammatory protein (MIP)-2, and monocyte chemoattractant protein (MCP)-1 expression levels were determined by real-time semiquantitative RT-PCR using SYBR universal PCR master-mix (Thermo Fischer Scientific, Waltham, MA) and the ABI PRISM 7700 (Applied Biosystems, Carlsbad, CA) sequence detection system, and analyzed as described in the online supplement.

Neutrophil Intracellular Signaling

Bone marrow–derived neutrophils were incubated with LPS (1 μg/ml), G-CSF (25 ng/ml), or PBS control for 15, 30, or 60 minutes. Neutrophils were lysed, total protein concentration of the supernatant was determined, and samples were analyzed by Western blot, as described in the online supplement.

Statistical Analysis

Data are presented as mean (±SEM), and analysis of differences between experimental groups was performed by Student *t* test. The difference between experimental groups over time was analyzed by two-way ANOVA. Associations between mouse weight and lung CFU levels were analyzed by linear regression. All analyses were performed using Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Results with a *P* value of 0.05 or less were considered statistically significant.

Results

Obesity Is Associated with Impaired Clearance of Bacteria in Mice

Obesity was found to worsen bacterial pneumonia in all four mouse models of obesity. However, differences were observed between bacterial burden and the temporal course of infection (Figures 1A–1D). Mice were examined at both 24 hours (Figures 2A–2P) and 48 hours (Figures 3A–3P) after infection. Bacterial counts at 24 hours after infection were increased in db/db, CPE $^{fat/fat}$, and ob/ob mice when compared with their lean littermates (Figures 2B–2D), and this increase in bacterial burden was significantly associated with increased body weight in these mice (Figures 2F–2H). No differences were observed in airspace neutrophilia between lean and obese mice from all models at 24 hours after infection (Figures 2I–2L). A significant difference in spleen bacterial counts was only observed between lean and obese ob/ob mice (Figures 2M–2P). Interestingly, DIO mice appeared to be able to contain the infection in the first 24 hours (Figures 2A and 2E), yet lung bacterial counts were increased at 48 hours after infection, which were also significantly associated with increased body weight (Figures 3A and 3E). The observed differences at 24 hours after infection between obese and lean lung bacterial counts in the CPE$^{fat/fat}$ and ob/ob models were no longer evident at 48 hours after infection (Figures 3C and 3D); although lung CFU in lean mice increased from 24 to 48 hours after infection, CFU remained similar in the obese mice in these models. Bacterial burden in obese db/db mice at 48 hours after infection, as well as its association with body weight, remained significantly different compared with their lean littermates (Figures 3B, 3F, and 3J), whereas airspace neutrophil counts were increased in obese db/db mice at this time point (Figure 3J). In addition, spleen bacterial counts were increased in obese DIO, db/db, and CPE$^{fat/fat}$ mice compared with their lean littermates at this time point (Figures 3M–3O). Taken together, these results suggest that obesity-associated failure to contain bacterial growth after *K. pneumoniae* infection, although common to all four models, differs substantially in its kinetics between models.

Obesity Attenuates Airspace Neutrophilia during LPS-Induced Pneumonitis

To further examine neutrophil recruitment to the airspace in the absence of bacterial expansion, we used a sterile model of pneumonitis using aerosolized LPS.

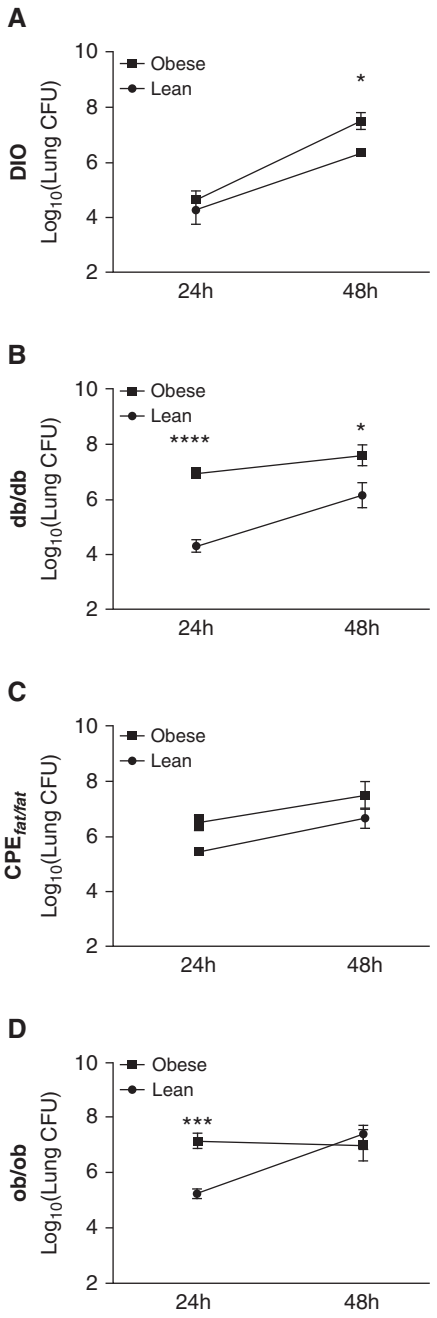

Figure 1. The course of bacterial burden in obese mice depends on the mouse model used. Lung colony-forming units (CFU) were determined at 24 and 48 hours after *Klebsiella pneumoniae* infection in (*A*) diet-induced obese (DIO) mice (60% fat diet versus 10% fat diet), (*B*) db/db mice, (*C*) CPE$^{fat/fat}$ mice, and (*D*) ob/ob mice and plotted over time. $n = 5$–13 mice/condition. Data are presented as mean (±SEM). *$P \leq 0.05$; ***$P \leq 0.01$; ****$P \leq 0.001$ compared with lean controls. A significant interaction between condition and time point two-way ANOVA was found in the ob/ob model ($P = 0.01$).

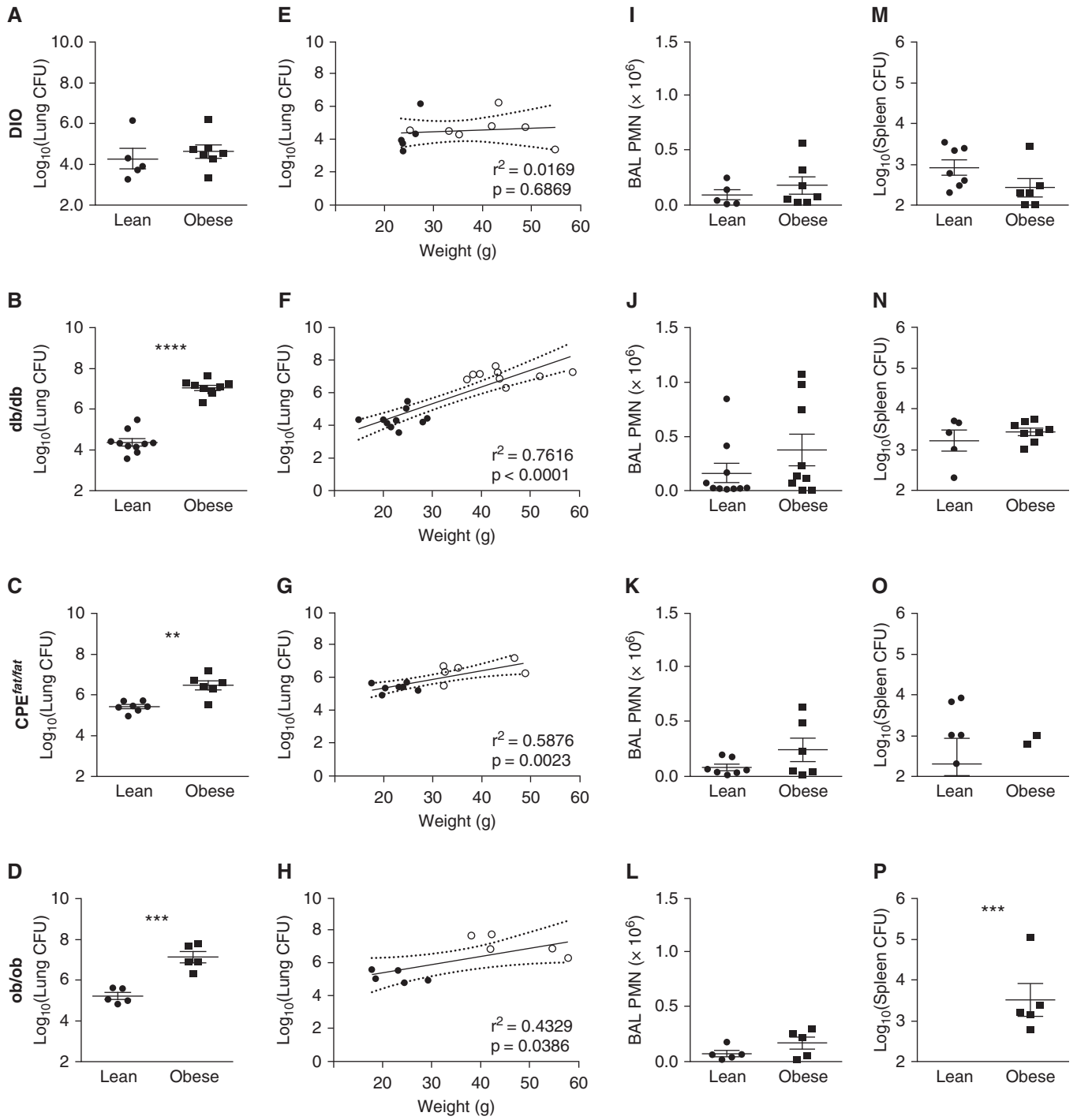

Figure 2. Obesity worsens bacterial pneumonia in mice. Lung CFU were determined at 24 hours after *K. pneumoniae* infection in (*A*) DIO mice (60% fat diet versus 10% fat diet), (*B*) db/db mice, (*C*) CPE$^{fat/fat}$ mice, and (*D*) ob/ob mice. (*E–H*) Lung CFU were compared with mouse weight by linear regression. In addition, (*I–L*) BAL neutrophil levels and (*M–P*) spleen CFU were determined. $n = 5$–13 mice/condition. *Dashed lines* indicate 95% confidence intervals. Data are presented as mean (±SEM). $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P < 0.0001$ compared with lean controls. *Solid circles*, lean mice; *open circles*, obese mice (*E–H*). BAL, bronchoalveolar lavage; PMN, polymorphonuclear cells.

Pulmonary neutrophilia was attenuated in the obese mice at 24 hours after LPS exposure in all models (Figures 4A–4D). The reduction in airspace neutrophil levels did not appear to be due to a decrease in neutrophil release from the bone marrow into the periphery in the DIO, db/db, and CPE$^{fat/fat}$ mice (Figures 4A–4C), as indicated by the elevated circulating neutrophil numbers. However, the obese ob/ob mice showed significantly lower peripheral neutrophil numbers

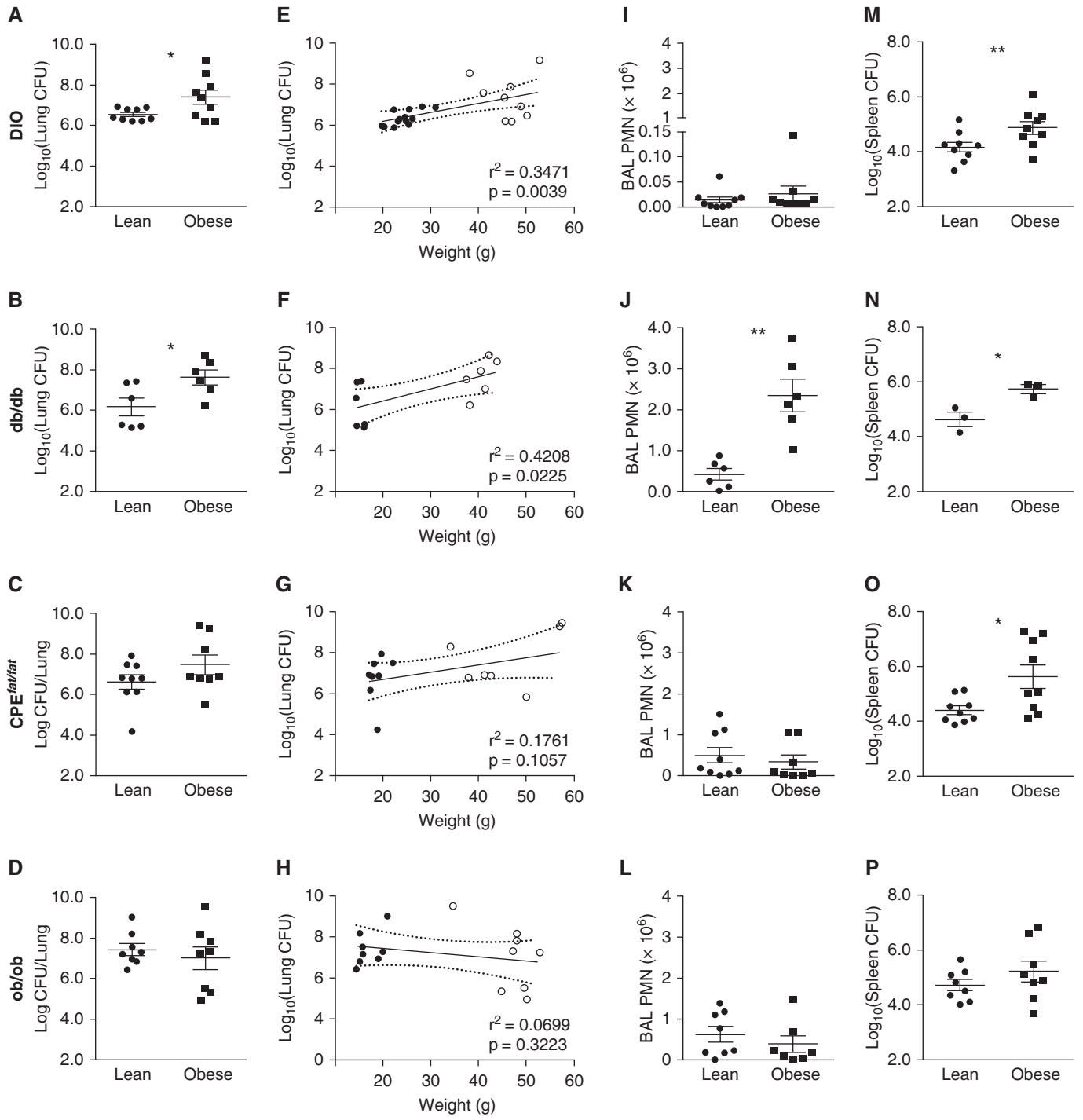

Figure 3. Obesity worsens bacterial pneumonia in mice. Lung CFU were determined at 48 hours after *K. pneumoniae* infection in (*A*) DIO mice (60% fat diet versus 10% fat diet), (*B*) db/db mice, (*C*) CPE$^{fat/fat}$ mice, and (*D*) and ob/ob mice. (*E–H*) Lung CFU were compared with mouse weight by linear regression. In addition, (*I–L*) BAL neutrophil levels and (*M–P*) spleen CFU were determined. $n = 5–13$ mice/condition. *Dashed lines* indicate 95% confidence intervals. Data are presented as mean (±SEM). $^*P \leq 0.05$, $^{**}P \leq 0.01$ compared with lean controls. *Solid circles*, lean mice; *open circles*, obese mice (*E–H*).

compared with their lean littermates (Figure 4D), indicating a different or possibly accompanying mechanism of decreased airspace neutrophilia. In addition, a significant decrease in alveolar cytokine levels (IL-6 and MCP-1) was observed at 24 hours after LPS in obese db/db and CPE$^{fat/fat}$ mice (Figures 4F and 4G), whereas no such differences were observed between lean and obese DIO and ob/ob mice (Figures 4E and 4H).

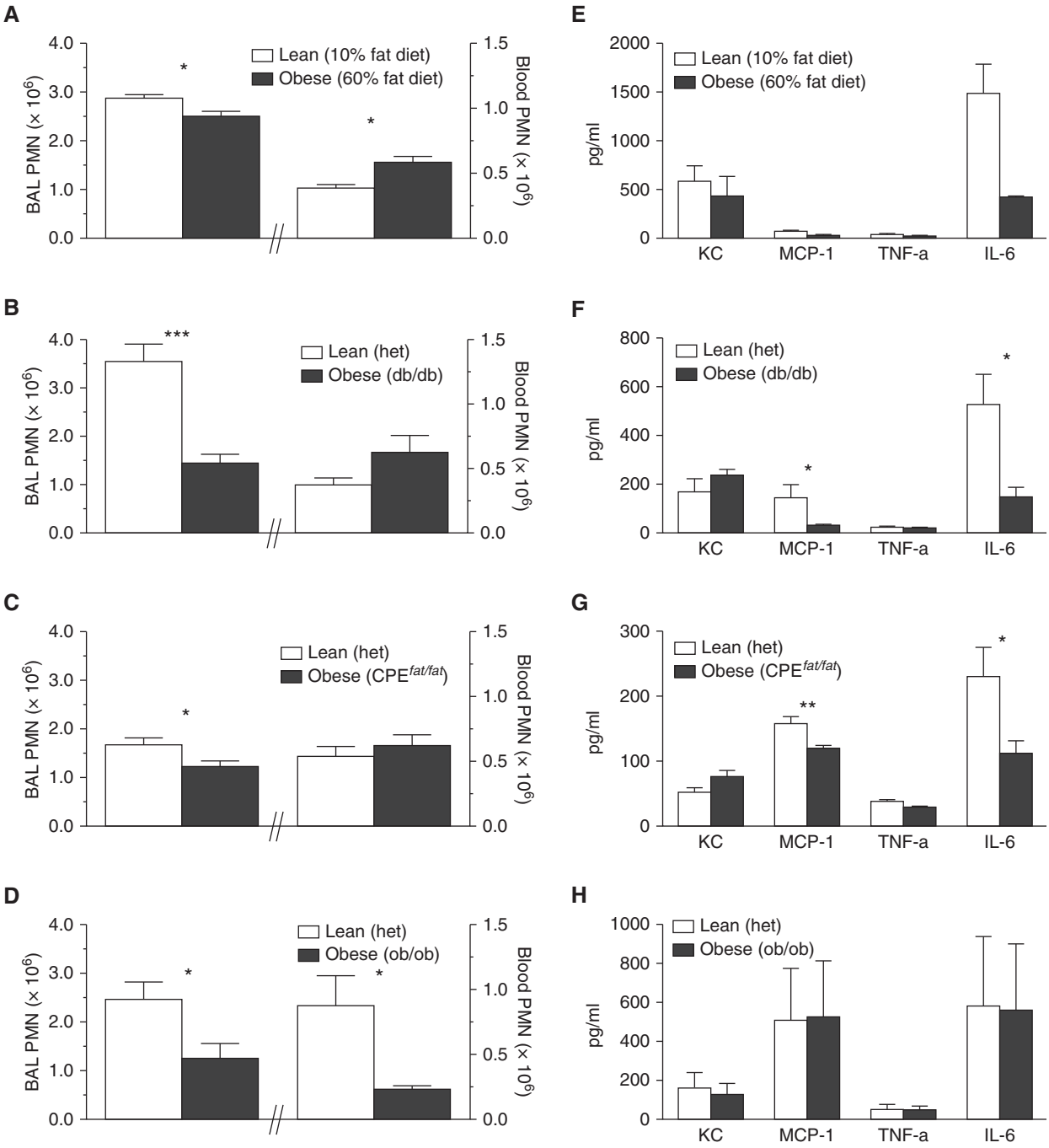

Figure 4. Obesity impairs the pulmonary immune response in mice after LPS-induced pneumonitis. (*A–D*) BAL and blood neutrophil counts and (*E–H*) BAL cytokine levels were determined in (*A* and *E*) DIO, (*B* and *F*) db/db, (*C* and *G*) CPE$^{fat/fat}$, and (*D* and *H*) ob/ob mice and their lean littermates at 24 hours after LPS exposure. $n = 8–12$ mice/condition. Data are presented as mean (±SEM). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with lean control. KC, keratinocyte chemoattractant; MCP, monocyte chemoattractant protein.

Obesity Impairs Neutrophil Chemotaxis

To determine whether the attenuated pulmonary neutrophilia observed in obese mice may be, at least in part, caused by an intrinsic defect in neutrophil function, we first examined the chemoattractant response of density-isolated, mature bone marrow–derived neutrophils from DIO, db/db, CPE$^{fat/fat}$, and ob/ob obese mice and their lean littermate controls. Chemotactic responses to KC (a CXCR2 ligand) and fMLP (a formyl peptide receptor 1 ligand) were impaired in neutrophils from obese DIO, db/db, CPE $^{fat/fat}$, and ob/ob mice (Figures 5A–5D) compared with their lean littermates, although, in ob/ob mice, the difference in chemotaxis response to fMLP did not reach statistical significance. These results indicate that obesity-associated defects in neutrophil chemotaxis are common among the examined

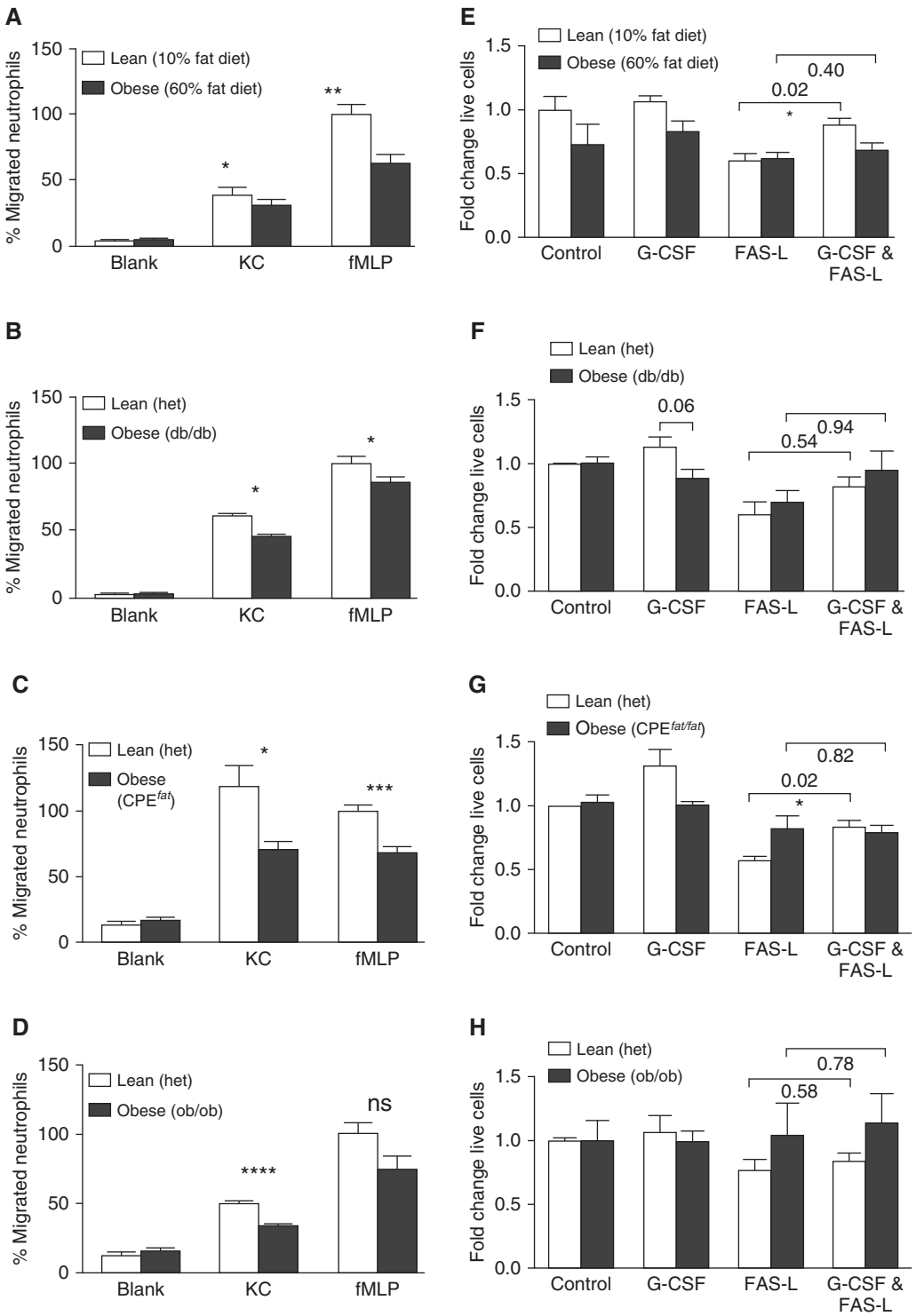

Figure 5. Obesity impairs neutrophil function. Chemotaxis of mature bone marrow neutrophils isolated from (*A*) DIO mice and (*B*) mutant obese db/db, (*C*) CPE$^{fat/fat}$, and (*D*) ob/ob mice was compared with lean control mice using a modified Boyden chamber with KC (25 ng/ml) or *N*-formylmethionyl-leucyl-phenylalanine (fMLP) (1 μM). Membrane counts were expressed as percentage of lean control neutrophil migration to fMLP for each experiment. Furthermore, the prosurvival effect of granulocyte colony-stimulating factor (G-CSF) on Fas ligand (FasL)-induced neutrophil apoptosis was examined. Mature bone marrow neutrophils isolated from (*E*) DIO mice and (*F*) mutant obese db/db, (*G*) CPE$^{fat/fat}$, and (*H*) ob/ob mice were incubated with G-CSF (25 ng/ml), FasL (200 ng/ml), or a combination of G-CSF and FasL for 6 hours. Live cells were then counted using trypan blue. Three separate experiments, using different mice for each experiment, were performed on isolated neutrophils from each mouse model and their respective controls. Cell counts were expressed as fold change of lean control live cell count for each experiment. Data are presented as mean (±SEM). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$ compared with lean control (*A–D*) or compared with G-CSF and FasL conditions (*E–H*). ns, not significant.

obesity models, and suggest that multiple G protein–coupled receptors are affected.

Obesity Variably Attenuates the Prosurvival Effect of G-CSF on FasL-Induced Apoptotic Neutrophils

The effects of obesity on neutrophil survival were determined by incubating mature bone marrow–derived neutrophils from each model of obesity with the antiapoptotic cytokine, G-CSF, proapoptotic, FasL, or a combination of both. Spontaneous apoptosis did not appear to be altered between lean and obese neutrophils from all models (Figures 5E–5H). Furthermore, neutrophils from lean mice of all models were susceptible to FasL-induced cell death, and could be rescued by G-CSF stimulation in lean controls from both DIO and CPE$^{fat/fat}$ models (Figures 5E–5H). However, neutrophils from obese DIO and CPE$^{fat/fat}$ mice demonstrated an attenuated prosurvival effect of G-CSF in the setting of FasL exposure. No differences were found in the number of viable cells between control and G-CSF–treated neutrophils from obese DIO, db/db, CPE$^{fat/fat}$, or ob/ob mice (Figures 5E–5H). None of these conditions appeared to have significant effects on neutrophils from obese ob/ob mice. These data suggest that neutrophils from obese DIO, db/db, and CPE$^{fat/fat}$ mice are not only defective in their chemotactic capacity, but may also have defects in survival. Thus, the obesity-associated attenuation of airspace neutrophilia in these models may derive from both impaired neutrophil recruitment and survival.

LPS-Induced Neutrophil Cytokine Transcription Is Variably Impaired in Obesity

To further delineate the effects of obesity on neutrophil function, we examined neutrophil cytokine transcription after LPS exposure. Bone marrow–derived neutrophils from obese mice and lean littermate controls were stimulated with LPS *in vitro* for 4 hours and mRNA expression levels of the cytokines IL-1β, MCP-1, IL-6, TNF-α, and KC were then determined. An overall decrease in cytokine production was observed in the obese neutrophils from DIO and db/db mice (Figures 6A and 6B) compared with lean littermate controls. However, no attenuation in cytokine expression was observed in neutrophils from obese ob/ob and CPE$^{fat/fat}$ mice (Figure 6D), and increased expression of TNF-α was seen in obese CPE$^{fat/fat}$ neutrophils compared with lean controls (Figure 6C). These results suggest that obesity may impair LPS-induced signaling, but that differences exist between the obese models studied.

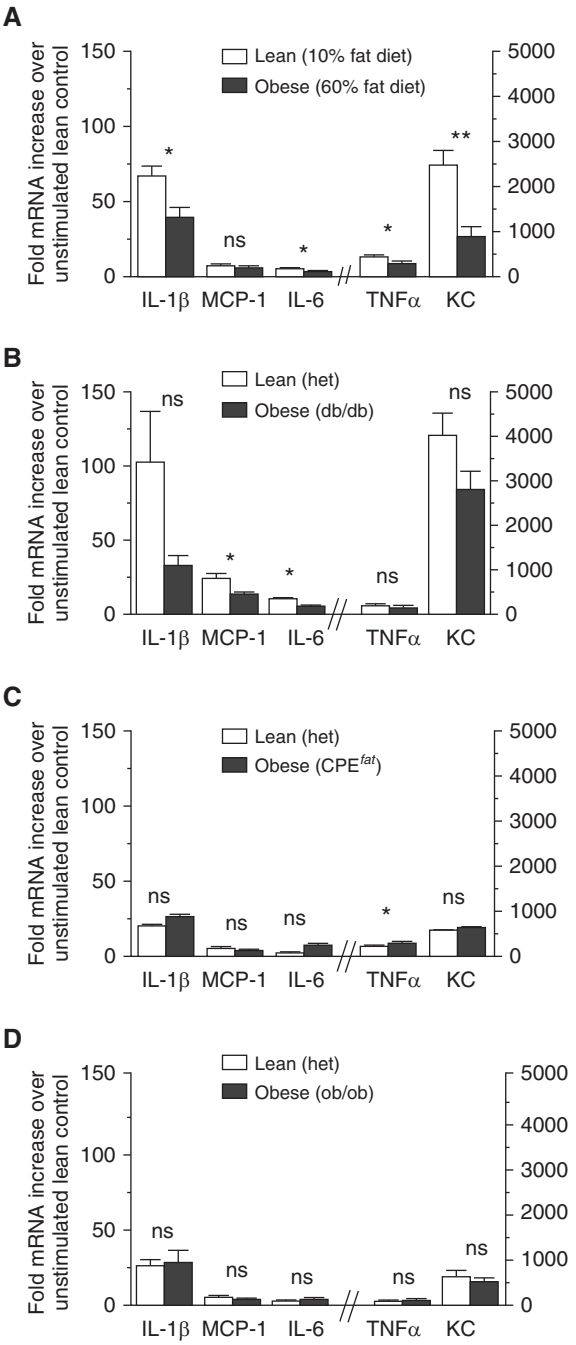

Figure 6. LPS-induced neutrophil cytokine transcription is variably impaired in obesity. Furthermore, gene expression levels of IL-1β, MCP-1, IL6, TNF-α, and KC levels were measured by quantitative PCR 4 hours after *in vitro* LPS (100 ng/ml) stimulation of bone marrow–derived neutrophils from (*A*) DIO, (*B*) db/db, (*C*) CPE$^{fat/fat}$, and (*D*) ob/ob obese mice and compared with lean control mice. Three separate experiments, using different mice for each experiment, were performed on isolated neutrophils from each mouse model and their respective controls. Data are presented as mean (±SEM). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ compared with lean control.

Obesity Impairs Neutrophil Signaling in Response to LPS and G-CSF

To examine the underlying mechanisms of the observed obesity-related neutrophil dysfunction, we next examined

neutrophil intracellular signaling. First, the mitogen-activated protein kinase signaling pathway was examined after LPS stimulation of bone marrow–derived neutrophils from obese DIO, db/db, CPE$^{fat/fa}$, and ob/ob mice and their lean littermates. An overall decrease in LPS-induced p38 phosphorylation was observed at various time points in neutrophils from obese DIO, db/db, and CPE$^{fat/fat}$ mice compared with their lean littermate controls (Figures 7A–7C). However, no distinct differences were observed between LPS-stimulated neutrophils from obese and lean ob/ob mice (Figure 7D). Next, we determined the downstream signaling response to G-CSF via signal transducer and activator of transcription (STAT) 3 signaling, which is important in directing neutrophilic granulocyte differentiation and cell survival (37, 38). G-CSF–induced signaling through STAT3 was reduced and possibly delayed in obese neutrophils from DIO and db/db mice, but appeared to be normal, but delayed, in obese CPE$^{fat/fat}$ mice and normal in ob/ob mice (Figures 7E–7H). These results may, in part, explain the impaired neutrophil transcriptional response to LPS, as well as the failure of G-CSF–induced neutrophil survival in several models of obesity.

Discussion

The present study presents evidence of obesity-associated defects in host defense to bacterial pneumonia in mice. However, the course and severity of pulmonary infection appear to vary with the mouse model used. Furthermore, although the inflammatory response after LPS-induced pneumonitis was attenuated in obese mice from all models, the mechanisms underlying this response appear to differ. We demonstrate that obesity is associated with variable defects in neutrophil functions, including chemotaxis, cell survival, and cytokine transcription, as well as impaired intracellular signaling responses.

Several animal models of obesity have been used to investigate the effects of obesity and associated comorbidities on the pulmonary immune response related to both acute and chronic lung diseases, including bacterial (11, 12, 18, 19, 27, 29) and viral (9, 28, 39, 40) respiratory infections, airway hyperresponsiveness (8, 41–46), ARDS (5, 7, 12, 30), pulmonary fibrosis (26), and chronic obstructive pulmonary disease (31). The most commonly employed mouse models of obesity include DIO, the hyperphagic mutant db/db, CPE $^{fat/fat}$, and ob/ob strains. DIO using high-fat–containing food in wild-type mice has been suggested to mimic human obesity most closely (20–22). However, most published reports use db/db mice (spontaneous mutants lacking expression of the long form of the leptin receptor, ObRb), which are typically considered to be a model of leptin "resistance" and obesity-associated diabetes (23). The obese, aleptinemic ob/ob mouse (a spontaneous mutant lacking expression of leptin) has also been used, particularly to determine the role and importance of leptin (24). Lastly, the CPE$^{fat/fat}$ mouse has been used as a general model of obesity, given its hyperphagic phenotype, which is driven by the lack of carboxypeptidase-E, which cleaves functional hormones regulating satiety (25).

Previous work using these models to investigate the effects of obesity on the susceptibility to and outcomes from bacterial respiratory infection has been inconclusive. Mancuso and colleagues (11, 18) showed that obese, leptin-deficient (ob/ob) mice exhibit increased susceptibility to infections with both gram-negative (*K. pneumoniae*) and gram-positive (Streptococcus *pneumoniae*) organisms, at 24 and 48 hours after inoculation, respectively, and that restoration of leptin levels could reverse the observed defects in bacterial clearance and survival (18). However, pulmonary host defense in hyperphagic CPE$^{fat/fat}$ mice (which manifest more modest metabolic abnormalities [25]) appeared to be normal at both 24 and 48 hours after

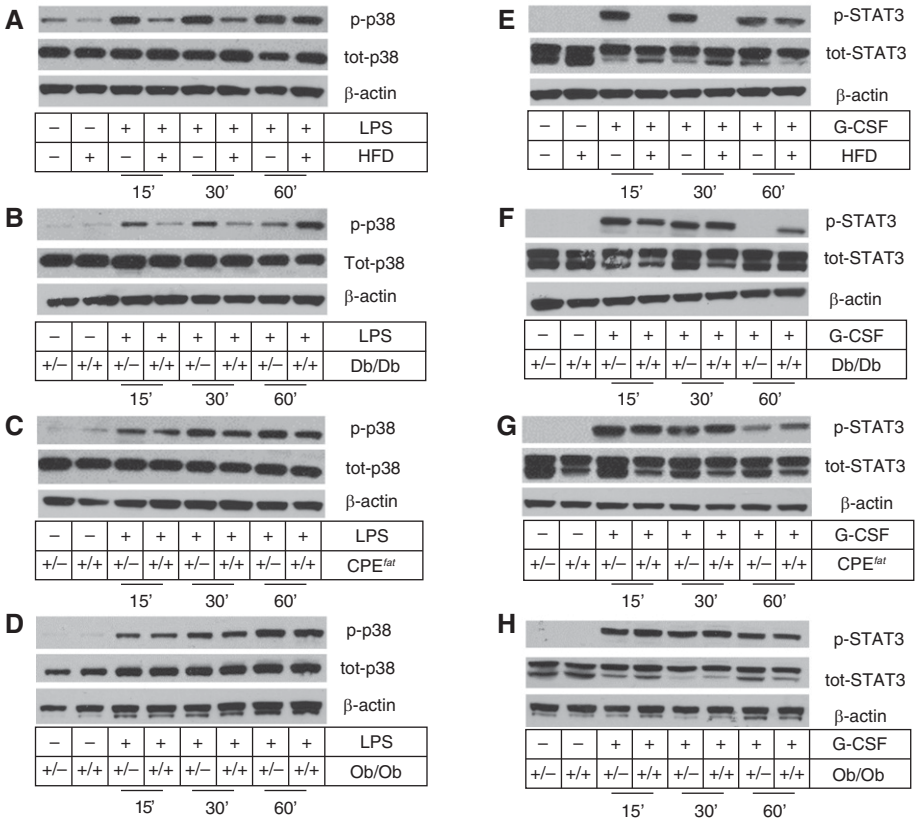

Figure 7. LPS-induced mitogen-activated protein kinase and G-CSF–induced signal transducer and activator of transcription (STAT) signaling response is attenuated in obese neutrophils. Bone marrow–derived neutrophils were isolated from (*A* and *E*) DIO (10 versus 60% fat diet) mice, (*B* and *F*) lean (+/−) and obese (+/+) db/db mice, (*C* and *G*) lean (+/−) and obese (+/+) CPE$^{fat/fat}$ mice, and (*D* and *H*) lean (+/−) and obese (+/+) ob/ob mice, and subsequently stimulated with PBS (control), (*A–D*) LPS (1 μg/ml) or (*E–H*) G-CSF (25 ng/ml) for 15, 30, or 60 minutes. Phosphorylated p38, total p38, phosphorylated STAT3, total STAT3, and β-actin (loading control) were determined by Western blot. Data shown are representative blots of three different experiments.

infection with *S. pneumoniae* (19). Here, we report evidence of impaired pulmonary host defense in response to *K. pneumoniae* in both ob/ob and CPE$^{fat/fat}$, as well as db/db and DIO, models of obesity, but with temporal variation. Early (24 h) lung bacterial burden was elevated in obese compared with lean db/db, ob/ob, and CPE$^{fat/fat}$ mice, but not in DIO mice. However, the effect of obesity was no longer evident at 48 hours in the ob/ob and CPE$^{fat/fat}$ models, whereas only becoming detectable at that time point in the DIO model, and remaining constant in the db/db model. The kinetic differences between these models may suggest the presence of either a "plateau" phenomenon, in which lung bacterial burden does not increase above a certain level (perhaps that corresponding to blood dissemination) in the obese mice, and/or differences in pulmonary bacterial handling in the early versus late phases of infection between the obesity models. Furthermore, we have previously shown that, although airspace neutrophil levels in response to *K. pneumoniae* infection are not significantly different between lean and obese db/db mice at 24 hours, there is a marked increase in bronchoalveolar lavage cytokine levels in obese db/db mice compared with their controls (12). This suggests that, in *K. pneumoniae*–infected obese mice, additional neutrophil recruitment signals are released as the infection worsens. Here, we report that airspace neutrophil levels are increased (in the obese db/db mice) at 48 hours after infection, but these neutrophils do not appear to be able to contain bacterial growth. The current findings, in the context of previous reports, suggest that obesity model, timing of observation, and the strain of bacteria used may lead to significantly different "outcomes" in obese pneumonia studies, despite a common obesity-associated susceptibility to pulmonary infection.

Our previous work suggests that an attenuated pulmonary inflammatory response may underlie the witnessed increased susceptibility to pulmonary infection in both subjects with obesity and obese animals (4, 5). In the current study, we find decreased pulmonary neutrophilia in all four murine obesity models after LPS-induced lung injury. However, our results also suggest that the underlying mechanisms may differ between these models. In the DIO, db/db, and CPE$^{fat/fat}$ models, circulating neutrophil counts after injury are similarly elevated between lean and obese mice, indicating that the attenuation of pulmonary neutrophilia is not due to a decrease in neutrophil release from the bone marrow to the periphery. However, we observed a decrease in circulating neutrophil levels in obese ob/ob mice, suggesting that a different or possibly accompanying mechanism may account for attenuated pulmonary neutrophilia in this model. In support of this, Claycombe and colleagues (47) previously reported that obese ob/ob (leptin-deficient) mice demonstrate impaired granulopoiesis at baseline, and that leptin replacement can reverse this defect. Thus, in the ob/ob model, defective granulopoiesis may, in part, underlie the impaired development of pulmonary neutrophilia after insult. Similar defects in granulopoiesis have not, however, been described in the other models of obesity, and, in fact, the opposite has been reported in the other mouse models of obesity and humans with obesity in which both resting and inflammatory states are associated with elevated blood neutrophil levels, possibly related to the effects of dyslipidemia and lipid overload (48–50).

Subsequent to blood delivery, neutrophil migration from the microvascular beds of the lung to the airspace is a crucial process after a pulmonary inflammatory insult. Previously, we reported obesity-associated impairment of neutrophil chemotaxis in the setting of blunted calcium flux response to the CXC cytokine KC (a homolog of human IL-8) and decreased surface levels of its receptor, CXCR2, on neutrophils in both DIO and db/db mice compared with their lean littermates (5). In the current study, we extend these findings to demonstrate defects in neutrophil chemotaxis across all four models of murine obesity and to both chemokines and bacterial peptides, suggesting that multiple G protein–coupled receptors (CXCR2 and formyl peptide receptor 1) are affected in the obesity-associated chemotaxis defect.

Another critical neutrophil function regulating levels of airspace neutrophilia and the pulmonary inflammatory response is neutrophil apoptosis. However, very little is known regarding the effects of obesity on neutrophil apoptosis. In the current study, we examined the effects of obesity on G-CSF–mediated rescue from FasL-induced neutrophil apoptosis, and found variable, but overall impaired, responses in obese mouse models, suggesting obesity-associated defects in G-CSF–mediated neutrophil survival. Perhaps underlying these findings, G-CSF–mediated STAT3 signaling response was reduced in obese neutrophils from DIO, db/db, and CPE$^{fat/fat}$ mice, whereas it appeared to be normal in ob/ob mice. Interestingly, no significant differences were observed in spontaneous neutrophil apoptosis between lean and obese mice from all models, suggesting that obesity may affect only induced, and not constitutive, neutrophil apoptosis. Together, these results suggest that the obesity-associated attenuation of airspace neutrophilia in these models may derive from both impaired neutrophil recruitment and, in some models, survival.

Beyond neutrophil transit to and survival in the lung, impaired neutrophil function may have other detrimental effects on the immune response to pathogens. The transcriptional response of neutrophils is a critical factor in amplifying pulmonary inflammation (51). Our current studies demonstrate an overall decrease in LPS-induced inflammatory cytokine transcription in neutrophils from both DIO and db/db models of obesity, yet little effect was seen in either CPE$^{fat/fat}$ or ob/ob models, suggesting that obesity may differentially impair LPS-induced signaling, depending on the model used. In support of this, we found that downstream signaling responses to LPS through the mitogen-activated protein kinase pathway were decreased in neutrophils from obese DIO mice, as well as db/db mice, whereas no distinct differences were seen when examining neutrophils from ob/ob mice. Subtle attenuation of LPS-induced p38 phosphorylation did not correlate with transcriptional impairment in obese CPE$^{fat/fat}$ neutrophils.

There are potential limitations to our approach worth noting. It has previously been reported that mutations in leptin production or signaling may result in congenitally smaller lungs in mutant (db/db and ob/ob) mice (52). In the current study, a "weight-neutral" approach to bacterial dosing was used with similar bacterial inocula being instilled in both lean and obese mice of all models. It is possible that, if the lungs of the ob/ob and db/db obese mice are indeed smaller, this may influence the rate and occurrence of bacterial overgrowth. Such an effect would not,

however, explain the differences seen in the $CPE^{fat/fat}$ or DIO models, in which lean and obese mice are known to have similar-sized lungs (52). Another possible confounder may be differences in the microbiome across the models, due to differing vendors and/or conditions. Our mutant obese animals and littermate controls were bred in our facility (and had been for at least five generations before use in these experiments), and it is known that the microbiota drift toward a facility-specific composition over time (53). Because the mutant mice were all cohoused in the same facility, no significant differences would be expected between lean and obese mice from these models. The possibility of significant differences between the microbiomes of the high-fat and low-fat diet mice in the DIO model is, however, significant, given the necessary lack of cohousing and the substantial differences in diet composition between the two groups. Thus, the witnessed differences between diet-induced lean and obese mice in our experiments may indeed reflect, at least in part, differences in their respective microbiomes. Similarly, sex differences may also affect the outcomes reported in the current study; however, both male and female mice were used in our experiments, and no differences were found in our readouts between male and female mice. Lastly, the potential exists for "off-target" effects of the obesity-associated mutations examined (ObRb deficiency and CPE deficiency) on neutrophil function in these models. To examine this possibility, we assayed neutrophil expression of these genes in wild-type mice and found that neutrophils lack both ObRb and CPE expression (*see* Figures E1A and E1B in the online supplement); however, as we have previously suggested (12), neutrophils do express the short form of the leptin receptor (ObRa) (Figure E1A), which has been shown to have signaling capacity (54). Thus, neither mutant model appears likely to have a direct, obesity-independent effect on neutrophil behavior.

Suitable animal models are fundamental to our understanding of the effects of obesity on pulmonary immune function and, through extrapolation, the pathogenesis of pulmonary disease in the population of humans with obesity. All obese mouse models integrate variable degrees of different elements of the metabolic syndrome, and therefore are imperfect models of individual facets of the metabolic syndrome (e.g., db/db mice as a model of simple diabetes). Factors contributing to the metabolic syndrome, including glucose intolerance and dyslipidemia, differ between the four models we examined (Table E1), likely leading to varying effects on the immune response and host defense. Diabetes and hyperglycemia have been suggested to increase the risk for and severity of infection (55), in line with our current results in the db/db model, which demonstrate both the greatest glucose intolerance (Table E1) and the most impaired ability to contain lung infection. Dyslipidemia has also been shown to impair both neutrophil function and the response to pneumonia (56, 57). Thus, the differing levels of hypercholesterolemia manifested in the four mouse obesity models (Table E1) likely also contribute to the variable degrees of neutrophil functional impairment and responses to pulmonary infection across these four models. Other potential factors that may contribute to the observed variability between mouse models in this study include genetic background, adipokine milieu, and the age at which obesity onset occurs. Obesity develops late in DIO mice (±24–28 wk), whereas db/db and ob/ob mice are obese by 8–10 weeks of age, and it has been suggested that older mice may respond differently to bacterial infections compared with younger mice (58). However, multivariate analysis, including both sex and age, has shown no effect of these variables on airspace neutrophilia after lung injury in our previous studies (5). Taken together, it is evident that, to select appropriate modeling approaches to obesity, a thorough understanding of the presence and degree of metabolic and other relevant factors manifest in each available model is essential to both interpret and maximize the clinical relevance of studies performed in these models.

Conclusions

We demonstrate that obesity is associated with impaired pulmonary host defense after bacterial infection. However, the course and severity of the infection, as well as the underlying mechanisms, may vary with the mouse model of obesity used (summarized in Table 1), likely on the basis of variable expression of the metabolic syndrome, as well as other factors discussed previously here. Furthermore, we show that obesity is associated with defects in neutrophil function, including impaired cytokine transcription, downstream signaling responses, and chemotaxis. The current work adds to our understanding of how results obtained in these different models should be interpreted, and to what extent metabolic parameters may vary between models. Given our growing appreciation of the Western diet's role in obesity pathogenesis, the DIO model appears to best recapitulate the human situation in which obesity is induced by an excessive intake of high-fat–containing food. However, the extent to which the factors associated with obesity and the metabolic syndrome are altered in this approach should be assessed when using this model, as variations in diet duration and composition, as well as genetic background, may alter these factors and thereby influence the study outcomes. Furthermore, the genetic abnormalities present in the mutant mouse obesity models examined are extremely rare in humans. Therefore, caution is recommended when extrapolating data generated in these particular obese animal models to the human condition. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Table 1. Overview of Outcomes per Model

	DIO	db/db	ob/ob	CPE^{fat}
LPS exposure: BAL PMN	↓↓	↓↓	↓	↓
LPS exposure: blood PMN	↑	↔	↔	↓
Klebs exposure: CFU 24 h	↔	↑↑↑	↑	↑↑
Klebs exposure: CFU 48 h	↑↑↑	↑↑↑	↔	↔
Chemotaxis	↓	↓	↓	↓
Cytokine transcription	↓	↓	↔/↑	↔
Signaling to G-CSF	↓↓	↓↓	↓	↔
Signaling to LPS	↓↓	↓↓	↓	↔

Definition of abbreviations: BAL, bronchoalveolar lavage; CFU, colony-forming units; DIO, diet-induced obesity; G-CSF, granulocyte colony-stimulating factor; PMN, polymorphonuclear cells.

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