Obesity Increases Mortality and Modulates the Lung Metabolome during Pandemic H1N1 Influenza Virus Infection in Mice

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Obesity Increases Mortality and Modulates the Lung Metabolome during Pandemic H1N1 Influenza Virus Infection in Mice

J. Justin Milner,* Jenny Rebeles,* Suraj Dhungana,† Delisha A. Stewart,† Susan C. J. Sumner,‡ Matthew H. Meyers,* Peter Mancuso,‡ and Melinda A. Beck*

Obese individuals are at greater risk for hospitalization and death from infection with the 2009 pandemic H1N1 influenza virus (pH1N1). In this study, diet-induced and genetic-induced obese mouse models were used to uncover potential mechanisms by which obesity increases pH1N1 severity. High-fat diet–induced and genetic-induced obese mice exhibited greater pH1N1 mortality, lung inflammatory responses, and excess lung damage despite similar levels of viral burden compared with lean control mice. Furthermore, obese mice had fewer bronchoalveolar macrophages and regulatory T cells during infection. Obesity is inherently a metabolic disease, and metabolic profiling has found widespread usage in metabolic and infectious disease models for identifying biomarkers and enhancing understanding of complex mechanisms of disease. To further characterize the consequences of obesity on pH1N1 infection responses, we performed global liquid chromatography–mass spectrometry metabolic profiling of lung tissue and urine. A number of metabolites were perturbed by obesity both prior to and during infection. Uncovered metabolic signatures were used to identify changes in metabolic pathways that were differentially altered in the lungs of obese mice such as fatty acid, phospholipid, and nucleotide metabolism. Taken together, obesity induces distinct alterations in the lung metabolome, perhaps contributing to aberrant pH1N1 immune responses. The Journal of Immunology, 2015, 194: 000–000.

*Department of Nutrition, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; †Systems and Translational Science Center, RTI International, Research Triangle Park, NC 27709; ‡Department of Environmental Health Sciences, University of Michigan, Ann Arbor, MI 48109

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Address correspondence and reprint requests to Dr. Melinda A. Beck, Department of Nutrition, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, 2303 MBRC, CB # 7461, Chapel Hill, NC 27599-7461. E-mail address: melinda.beck@unc.edu

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Abbreviations used in this article: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; CD, chow diet; dpi, day postinfection; HFID, high-fat diet; Lepr<sup>−/−</sup> mice, with disruption of leptin receptor signaling in hypothalamic neurons; Lepr<sup>-/Hi</sup>, heterozygous leptin receptor mice; Lepr<sup>+/Hi</sup>, fully fixed leptin receptor mice; LFD, low-fat diet; mLN, mediastinal lymph node; MS, mass spectrometry; OPLS-DA, orthogonal partial least squares discriminant analysis; pH1N1, 2009 pandemic H1N1 influenza A virus; A/California/04/2009; TAG, triacylglycerol; TCID<sub>50</sub>, 50% tissue culture infective dose; Treg, regulatory T cell; UPLC, ultra-performance liquid chromatography; UPLC-MS, ultra-performance liquid chromatography–mass spectrometry.

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the global lung metabolome (at the site of infection) or how altered systemic metabolism (e.g., obesity) may impact influenza pathogenesis and metabolic processes in the lung.

In this study we used two models of obesity, diet- and genetic-induced, providing a robust characterization of the immunological and metabolic consequences of obesity during pH1N1 infection. High-fat diet (HFD)-induced and genetic-induced obese mice exhibited greater pH1N1 mortality, as well as elevated lung inflammatory responses and excess lung damage, despite similar viral titers compared with lean control mice. Additionally, both models of obesity exhibited distinct alterations in immune cell populations, such as fewer macrophages and regulatory T cells (Tregs) in the airways. We also demonstrated that the lung metabolome was differentially altered by obesity during influenza virus infection. Furthermore, ultra-performance liquid chromatography–mass spectrometry (UPLC-MS) profiling successfully distinguished urine samples from infected lean and obese mice as early as 2 d postinfection (dpi), and the urine from infected obese mice reflected alterations in a diverse number of metabolic pathways. Pathway enrichment analyses, based on the uncovered metabolic signatures in lung tissue and urine, revealed differentially regulated metabolic processes that perhaps may be contributing to greater pH1N1 severity in obese mice, such as fatty acid, phospholipid, and nucleotide metabolism. Taken together, the present study provides an in-depth analysis of the immunological and metabolic consequences of obesity during influenza virus infection.

Materials and Methods

Mice and diets

Diet-induced obesity was achieved by maintaining weanling, male C57BL6/J mice (obtained from The Jackson Laboratory, Bar Harbor, ME) on a HFD (60% kcal fat, Research Diets, New Brunswick, NJ, D12492 formula), and lean mice were maintained on a low-fat diet (LFD; 10% kcal fat, Research Diets, D12450B formula) or a standard chow diet (CD; 14% kcal fat, ProLab RMH 3000, LabDiet, St. Louis, MO) for 14–16 wk. The HFD and LFD are nutritionally defined and contain purified ingredients. The HFD and LFD are nutritionally matched except for fat content and carbohydrate content (70% kcal in LFD and 20% kcal in HFD provided by carbohydrates). For the CD, calories are derived from 26% protein, 14% fat, and 60% carbohydrate.

Genetically induced obesity was achieved by crossing fully floxed leptin receptor mice on a C57BL6/J background (provided by Dr. Alyssa Hasty) with C57BL6/J-Tg(Nkx2-1-cre)2Sand/J mice purchased from The Jackson Laboratory. Fully floxed mice expressing the Cre transgene under control of the Nkx2.1 promoter (LeprRn/+) lack leptin receptor signaling in hypothalamic neurons (LeprR−/−) and become obese owing to hyperphagia (32). Heterozygous breeding using LeprR+/+ and LeprR−/− mice was necessary because homozygous LeprR−/− mice do not produce offspring (32). Mice were genotyped as previously described (32) and housed in isolation cubicles at the University of North Carolina, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. All procedures involving the use of mice were fully approved by the University of North Carolina Institutional Animal Care and Use Committee.

Influenza virus infection and viral titers

Influenza A/California/04/2009 (BEI Resources, Bethesda, MD) was propagated in embryonated hen’s eggs and titered via a modified 50% tissue culture infective dose (TCID50) using hemagglutination as an endpoint and evaluated by the method of Reed and Muench (33) as previously described (8, 12). Mice were lightly anesthetized via isoflurane inhalation and were infected intranasally with 0.05 ml of 5.8 ± 10^2 TCID50 or 1.3 ± 10^3 TCID50 as described in the figure legends. For diet-induced obese mouse models, mice were infected at 17–19 wk of age. LeprR+/+ and LeprR−/− mice were infected at 12–25 wk of age (LeprRn/+ and LeprR−/− were age matched). For influenza virus titers of infected mice, bronchoalveolar lavage fluid (BALF) was titered via a modified TCID50 in replicates of four.

Serum and BALF measurements

BALF was obtained as previously described (14). ELISA kits were used to measure serum leptin (Invitrogen, Carlsbad, CA), triacylglycerol (TAG; Pointe Scientific, Canton, MI), insulin (Merckodia, Uppsala, Sweden), and adiponectin (Abcam, Cambridge, U.K.). Blood glucose was measured via a 6- or 14-h fast as described in the figure legends. Furthermore, BALF albumin was measured with a mouse albumin ELISA kit (GenWay Biotech, San Diego, CA). Total protein in BALF was measured via a standard BCA assay (BCA kit, Sigma-Aldrich, St Louis, MO). BALF cytokines (IL-4, IFN-γ, MCP-1, RANTES, KC, IL-17A, IL-10, and TNF-α) were measured using a Bio-Plex assay (Bio-Rad, Hercules, CA) per the manufacturer’s instructions. IL-4 was not measurable above the lower limit of the assay.

Lung histopathology

The left lobe of the lung was inflated and fixed with 10% neutral buffered formalin. Fixed lung tissue was embedded in paraffin and processed for H&E staining by the University of North Carolina Animal Histopathology Core Facility. The extent of lung immune cell infiltration was scored blindly according to a scale from 0 to 4: 0, no inflammation; 1, mild influx of inflammatory cells; 2, increased inflammation with ~25–50% of the total lung involved; 3, severe inflammation involving 50–75% of the lung; and 4, almost all lung tissue contains inflammatory infiltrate (12, 14).

Flow cytometry

Lung, mediastinal lymph nodes (mLN), and BAL cells were stained for flow cytometry as previously described (14). For staining of BAL cells from uninfected mice, BAL samples were pooled from two individual mice to obtain a sufficient number of cells (two pooled BAL samples represent n = 1). The following Abs were used: CD4 (FTTC, PE-Cy7), CD103 (PE), F4/80 (allophycocyanin), Ly6G (FTTC), NK1.1 (PE-Cy7), and F4/80 (allophycocyanin) from ebioscience (San Diego, CA); CD8 (PerCP) and CD3 (allophycocyanin) from Biolegend (San Diego, CA); and CD25 (FITC) from BD Biosciences (San Jose, CA). For intracellular staining, all cells were fixed and permeabilized using a Fcosp3 staining buffer kit (ebioScience). All samples were run on a CyAn ADP analyzer flow cytometer (Beckman Coulter, Fullerton, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR). All cells were analyzed by first utilizing a doublet exclusion gate followed by gating of CD3+ T cells for further analysis of CD4+ and CD8+ T cell populations or by gating on CD3+ and CD103+ T cell populations for further analysis of macrophages (F4/80+), neutrophils (Ly6G+), or NK cells (NK1.1+).

Metabolic profiling

Lung tissue was harvested and immediately frozen in liquid nitrogen. Urine was collected as previously described (25). To every 10 mg lung tissue 100 μl 50:50 acetonitrile/water was added and the tissue was homogenized using a bead beater operating at 1750 rpm. A 300-μl sample of lung homogenate was spiked with tryptophan-d3 internal standard, lyophilized, reconstituted in 100 μl 95:5 water/methanol, and 10 μl was injected into a SYNAPT G2 quadrupole time-of-flight mass spectrometer coupled to an Acquity UPLC (Waters, Milford, MA) for broad-spectrum metabolomics analysis. The lung metabolites were separated on a Waters Acquity BEH HSS T3 column (2.1 × 100 mm, 1.8 μm particle size) operating at 50°C using a reversed-phase chromatographic method. A gradient mobile phase consisting of water with 0.1% formic acid and methanol with 0.1% formic acid were used as previously described (34). Urine samples (25 μl) were diluted with acetonitrile containing tryptophan-d3 internal standard (75 μl). A 5 μl diluted urine was injected into a SYNAPT G2 quadrupole time-of-flight mass spectrometer coupled to an Acquity UPLC (Waters) for broad-spectrum metabolomics analysis. The urine metabolites were separated on a Waters Acquity BEH amide column (2.1 × 150 mm, 1.7 μm particle size) operating at 40°C using a hydrophilic interaction chromatography method. A gradient mobile phase containing 10 mM ammonium acetate in 95:5 acetonitrile/H2O with 0.1% formic acid (A) and 10 mM ammonium acetate in 50:50 acetonitrile/H2O with 0.1% formic acid (B) was used under the following conditions: 0–1.0 min, 1% B; 1.0–12.0 min, 100% B; 12.0–15.0 min, 1% B; 15.0–19.0 min, 1% B. All MS data were collected over 50–1000 m/z in electrospray ionization positive and negative ion modes. Leucine enkephalin was used as the lock mass and a lock mass scan was collected every 4 s and averaged across three scans to perform mass correction. Source and desolvation temperatures were set at 110°C and 450°C, respectively. All MS data analyses (alignment, normalization, and peak picking) were performed using Progenesis QI (Waters). Multivariate analysis (principal component analysis and orthogonal partial least squares discriminant analysis [OPLS-DA]) of the metabolomics data were performed using SIMCA (Umetrics, Umeå, Sweden) (35). Putative identification of the group differentiating metabolites was made using a database search against RTI International–Eastern Regional Comprehensive Metabolomics Resource Core’s in-house exact mass retention time library of standards (~500 compounds) and Human...
were considered statistically significant at a TCID\textsubscript{50} of the 2009 pandemic H1N1 virus, influenza A/Cal/04/09.

To further investigate the consequences of obesity pathology, alters lung immune cell populations, impairs lung healing, and in some cases increases viral titers during influenza infection in mice (10–15, 36). To investigate the sequels of obesity on pH1N1 immunity, weanling male C57BL6/J mice were fed a purined LFD (10% kcal fat, nutritionally matched to the HFD except for fat and carbohydrate content), or a standard CD (14% kcal fat). The two low-fat control diets (LFD and CD) were included because although previous studies have used both of these diets as controls for a HFD, they have never been directly compared with each other in the same study (10–15, 36). As expected, mice fed a HFD gained significantly more weight compared with LFD- and CD-fed mice (Fig. 1A). Although LFD and CD mice exhibited relatively similar levels of weight gain in comparison with HFD-induced obese mice, LFD mice weighed significantly more than CD mice after 8 wk on the diet. Obesity results in metabolic perturbations, reflected by alterations in a number of circulating hormones and nutrients (16). In Fig. 1B, we demonstrate that HFD-fed obese mice had elevated fasting blood glucose measures compared with LFD and CD mice. Furthermore, serum insulin was elevated in HFD mice compared with CD mice, and LFD mice exhibited a relatively intermediate concentration. Additionally, HFD mice had a greater concentration of serum TAG and leptin but lower adiponectin levels. In summary, HFD mice exhibited prototypical characteristics of obesity, whereas LFD mice exhibited a somewhat intermediate phenotype compared with HFD and CD mice.

After 14–16 wk on the diet, mice were infected with 5.8 × 10\textsuperscript{3} TCID\textsubscript{50} of the 2009 pandemic H1N1 virus, influenza A/Cal/04/09 (pH1N1). Strikingly, although no CD mice succumbed to the pH1N1 infection, 40% of LFD mice and >80% of HFD mice died by 10 dpi (Fig. 1C). HFD-fed obese mice exhibited a significantly higher mortality rate compared with both LFD and CD mice. Although it has been shown that diet-induced obese mice are more susceptible to pH1N1 mortality (10), it has never been demonstrated that LFD-fed mice are also more likely to die of influenza infection compared with CD mice. Analysis of absolute weight loss and percentage weight loss demonstrated that CD mice recover faster from the infection compared with HFD and LFD mice (Fig. 1D, 1E).

Given that all CD mice survived the infection, we next tested whether the discrepancy in pH1N1 mortality between the three dietary groups was maintained with a greater pH1N1 dose. All three dietary groups were infected with 1.3 × 10\textsuperscript{5} TCID\textsubscript{50}, and by 7 dpi, 83% of HFD mice died (Fig. 1F) compared with 55% of LFD mice and only 33% of CD mice. Despite the increased death in all dietary groups compared with the previous lower dose, the mortality differences between the dietary groups were relatively maintained in that HFD and LFD mice were more susceptible to mortality compared with CD mice (\(p < 0.005\) and \(p = 0.05\), respectively), and HFD mice trended toward increased mortality compared with LFD mice (\(p = 0.08\)). There were no differences in total weight loss between the three groups (Fig. 1G), but HFD mice displayed a significantly lower percentage weight loss during the course of the infection compared with LFD and CD mice (Fig. 1H), which has been reported previously (10). In summary, we show that HFD-induced obesity increases pH1N1 infection severity compared with two different lean mouse models. Given that LFD mice exhibited greater mortality than did CD mice, simply changing the components of a diet (without inducing any nutritional deficiencies) can modulate influenza infection severity, and thus we cannot rule out synergistic effects of a HFD and obesity.

### Results

#### Diet-induced obese mice are more susceptible to pH1N1 mortality compared with CD-fed and purified LFD-fed lean mice

Epidemiological evidence demonstrates that obesity increases the likelihood of severe influenza infection complications in humans (4, 6, 17, 37, 38), and several mouse models have also confirmed this outcome (10–15, 36). Obesity exacerbates lung inflammation and pathology, alters lung immune cell populations, impairs lung healing, and in some cases increases viral titers during influenza infection in mice (10–15, 36). To further investigate the consequences of obesity on pH1N1 immunity, weanling male C57BL6/J mice were fed a purified LFD (10% kcal fat, nutritionally matched to the HFD except for fat and carbohydrate content), or a standard CD (14% kcal fat). The two low-fat control diets (LFD and CD) were included because although previous studies have used both of these diets as controls for a HFD, they have never been directly compared with each other in the same study (10–15, 36).

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HFD-induced obese mice have fewer macrophages and Tregs in the lung airways compared with lean mice

To gain a better understanding of the causes and consequences of greater lung injury and alterations in chemokine expression in HFD mice, the distribution of innate immune cells in the airways of pH1N1-infected CD, LFD, and HFD mice was assessed. Fig. 3A is a representative flow cytometry histogram for identification of macrophages, neutrophils, and NK cells. Fig. 3B represents the percentages (top) and numbers (bottom) of F4/80+ macrophages, NK1.1+ NK cells, and Ly6G+ neutrophils. Unexpectedly, HFD mice had significantly fewer BAL macrophages at 4 dpi, trending fewer NK cell numbers \((p = 0.07)\) as well as similar numbers of neutrophils with CD mice. Although HFD mice had greater levels of KC and MCP-1 expression (neutrophil and macrophage chemokines, respectively), this did not result in greater infiltration of macrophages or neutrophils.

We then measured the distribution of CD4+ and CD8+ T cells in the BAL, lung, and mLN of the three dietary groups. HFD mice had fewer BAL CD4+ T cells at 8 dpi with no differences in CD8+ T cell numbers compared with CD mice (Fig. 3C, 3D). Given the alterations in T cell numbers detected in the lung airways, CD4+ and CD8+ T cell numbers were measured in lung tissue and in mLN at 4 and 8 dpi. There were no differences in lung or mLN T cell numbers between CD and HFD mice (data not shown).

CD4+Foxp3+ Tregs have been shown to curtail inflammatory responses to respiratory syncytial virus and some influenza infection models in mice (39–41). Because we detected fewer BAL CD4+ T cells in HFD mice, we also suspected fewer BAL Tregs as well, perhaps contributing to heightened lung inflammation and damage in HFD mice. Fig. 3E is a representative gating scheme for identification of BAL Tregs in CD, LFD, and HFD mice. HFD mice had a significantly greater percentage of Tregs at 4 dpi (Fig. 3E), and consistent with fewer CD4+ T cells at 8 dpi, HFD mice had fewer Tregs at 8 dpi (Fig. 3F). Additionally, the level of Treg activation via CD103 expression was measured (42), and HFD mice had fewer CD103+ BAL Tregs as well (Fig. 3G). Given the discrepancy in Treg number in the lung airways, Treg distribution in lung tissue and mLN were also assessed (Fig. 3H, 3I). HFD mice had fewer Tregs at 0 and 4 dpi in the lung tissue and fewer at 8 dpi in the mLN compared with LFD mice.

Metabolic profiling distinguishes lung samples from uninfected and infected obese mice

Obesity is inherently a metabolic disease, and metabolic profiling has found widespread usage in infectious disease models for identifying biomarkers and improving our understanding of these complex diseases (23–26). Therefore, we hypothesized that metabolic profiling would not only provide a snapshot of the metabolic consequences of obesity in the lung during pH1N1 infection, but it would also shed...
light on potential mechanisms driving greater pH1N1 severity in obese mice. Lungs were harvested from CD, LFD, and HFD mice at 0, 4, and 8 dpi, and UPLC-MS metabolic profiling was performed. In Fig. 4A, the OPLS-DA plot shows significant separation between lung samples from uninfected CD and HFD mice. As one might expect, several metabolites were altered by the HFD and/or obesity, and most of these metabolites were related to lipid metabolism (involved in fatty, cholesterol, or phospholipid metabolic pathways). Once infected, the HFD and CD lung metabolic profiles continued to separate (Fig. 4B). An increase in differential metabolites was observed in HFD infected mice at 4 dpi, particularly in phospholipid and nucleotide metabolites (Fig. 4B).

By 8 dpi, the number of metabolites significantly altered by obesity more than doubled compared with uninfected lung tissue (Fig. 4C). Fatty acid- and phospholipid-related species comprised most significantly altered metabolites in HFD mice at 8 dpi. Furthermore, several amino acid and ketone metabolites were detected at high levels in the lungs [with (S)-3-hydroxybutyric acid detected at a 17.5-fold greater concentration in lungs of HFD mice, \( p < 0.0005 \)]. Few of the differentially altered metabolites in uninfected HFD mice were also different at 8 dpi, indicating that the detected metabolic changes occurring in the lung during infection are not simply due to obesity or HFD feeding, but are a consequence of the infection in the HFD-induced obese mice.

Lastly, we determined whether the unique metabolic changes that were measured in the lungs of HFD mice at 8 dpi may provide a snapshot of some of the altered dynamic metabolic processes occurring during the infection. We used the GeneGo MetaCore systems biology software to uncover differentially altered metabolic pathways (Fig. 4D). The most significantly altered pathway in the lungs of obese mice at 8 dpi was N-acyltransferase metabolism (\( p < 0.0005 \)), as well as a number of fatty acid–related pathways and nucleotide metabolism pathways (Fig. 4D). For metabolite comparisons, we primarily focused on differences between

FIGURE 2. HFD mice exhibit greater pH1N1 lung damage and inflammation. (A) BALF viral titers after 5.8 \( \times 10^2 \) TCID\(_{50}\) pH1N1 infection (\( n = 4 \) for uninfected mice and \( n = 7–8 \) at 4 and 8 dpi). (B and C) Total number of BAL cells (B) and lung cells (C) (\( n \geq 8 \)). (D) H&E-stained lung pathology slides (\( n = 6–8 \)). Each pathology image represents a \( \times 40 \) magnification of the designated area shown in the \( \times 4 \) magnified image in the top right of each field. (E and F) Fold increase of BALF protein (E) (\( n = 6–10 \)) and BALF albumin (F) (\( n = 5–7 \)) during the pH1N1 infection. Fold increase was normalized to uninfected mice within each dietary group. (G) Fold change of BALF cytokine concentrations between CD, LFD, and HFD mice determined from a multiplex assay (\( n = 5–8 \)). Each bar or datum point represents mean ± SEM. \(* p < 0.05, ** p < 0.005 \) comparing HFD with CD mice; \( ^{*} p < 0.05 \) comparing HFD with LFD mice; and \( ^{#} p < 0.005 \) comparing CD with LFD mice.
CD and HFD mice because we detected the greatest discrepancy in infection severity between these two groups. However, a complete list of differentially altered metabolites between CD, LFD, and HFD mice as well as fold change and \( p \) values are listed in Supplemental Table I.

Obesity, independent of diet, increases pH1N1 mortality

HFD mice exhibited greater pH1N1 infection severity, alterations in inflammatory immune responses, and diverse changes in lung metabolism during the infection. However, a limitation of utilizing a diet model of obesity is that differential outcomes between lean (CD or LFD) and HFD obese mice could be influenced by differences in the diets, potentially confounding the effects of obesity. Furthermore, LFD mice display greater mortality compared with CD mice, suggesting that simply modulating the diet (without causing any nutritional deficiencies) can impact pH1N1 infection outcome, although not to the same extent as in HFD obese mice. Therefore, we included a genetic model of obesity to...
assess the impact of obesity on infection responses independent of dietary effects.

We used a genetic model of obesity in which excess adiposity is driven primarily by hyperphagia (all mice are fed a CD). It has previously been shown that ob/ob mice, a robust model of genetic obesity, are more susceptible to pH1N1 infection compared with wild-type mice (10). However, utilization of the ob/ob mouse model has a number of limitations due to the global deficiency of leptin signaling. Leptin is critical for proper physiology and immunity, and leptin deficiency has been shown to impair host defenses, thus confounding the immunomodulatory consequences of obesity (8). Therefore, we used a tissue-specific model, previously characterized by Ring and Zeltser (32), in which disruption of leptin signaling is limited to hypothalamic neurons (the primary site of leptin-mediated appetite control) to further address the impact of obesity on pH1N1 immunity. These mice become rapidly obese and exhibit hyperinsulinemia, hyperglycemia, and hyperleptinemia among other metabolic alterations characteristic of obesity (32).

Male (Fig. 5A) and female (Fig. 5B) LepRH$^{2/2}$ mice, lacking functional leptin signaling in hypothalamic neurons, rapidly gained excess body weight compared with lean LepRH$^{fl/fl}$ and LepRH$^{+/2}$ mice. At 13–16 wk of age, LepRH$^{2/2}$, LepRH$^{+/2}$, and LepRH$^{fl/fl}$ mice were infected with $5.8 \times 10^2$ TCID$_{50}$ pH1N1. Strikingly, obese male (Fig. 5C) and female LepRH$^{2/2}$ (Fig. 5D) mice were significantly more susceptible to pH1N1 mortality compared with lean LepRH$^{+/2}$ mice. Obese male and female LepRH$^{2/2}$ mice lost more absolute weight during the pH1N1 infection compared with both lean groups (Fig. 5E, 5F). Obese male and female LepRH$^{2/2}$...
mice displayed a lower percentage of body weight lost compared with lean LepRH<sup>H<sup>H</sup>/<sup>H</sup> mice, which is likely due to the greater preinfection weight of LepRH<sup>H<sup>RH</sup>2/2</sup> mice (n = 9). (C and D) Mortality curves for male (C) and female (D) mice postinfection with 5.8 × 10<sup>7</sup> TCID<sub>50</sub> pH1N1 at 14–16 wk of age (n = 9). (E and F) Total weight loss for male (E) and female (F) mice after pH1N1 infection (n = 9). (G and H) Percentage weight loss for male (G) and female (H) mice after pH1N1 infection (n = 9). Each datum point represents mean ± SEM. In (A)–(F), *p < 0.05, **p < 0.005, or ***p < 0.0005 comparing LepRH<sup>H<sup>RH</sup>2/2</sup> with both LepRH<sup>H<sup>RH</sup>2/2</sup> and LepRH<sup>H<sup>RH</sup>+/2</sup> mice; for (G) and (H), **p < 0.05 or ***p < 0.0005 comparing LepRH<sup>H<sup>RH</sup>2/2</sup> with LepRH<sup>H<sup>H</sup>/fl/fl</sup> mice and *p < 0.05 or **p < 0.005 comparing LepRH<sup>H<sup>RH</sup>2/2</sup> with LepRH<sup>H<sup>H</sup>/fl/fl</sup> mice.

Obese LepRH<sup>H<sup>RH</sup>2/2</sup> mice exhibit greater lung inflammatory responses and alterations in lung immune cell populations during pH1N1 infection

Given the elevated mortality in the LepRH<sup>H<sup>RH</sup>2/2</sup> obese mice compared with lean controls, we next assessed pathological and immunological responses in the lungs of LepRH<sup>H<sup>RH</sup>2/2</sup> mice. Fig. 6A demonstrates that whereas LepRH<sup>H<sup>RH</sup>2/2</sup> mice had significantly larger mesenteric white adipose tissue depots and livers compared with lean LepRH<sup>H<sup>H</sup>/fl/fl</sup> mice, there were no differences in lung weight. Lung viral titers were then measured, and LepRH<sup>H<sup>RH</sup>2/2</sup> did not exhibit differences in viral burden (Fig. 6B). However, the obese LepRH<sup>H<sup>RH</sup>2/2</sup> mice did have fewer infiltrating cells into the airways during the infection (Fig. 6C), but they exhibited a greater level of injury to the lung epithelium compared with lean LepRH<sup>H<sup>H</sup>/fl/fl</sup> mice at 8 dpi (Fig. 6D). Gene expression of lung cytokines and chemokines also demonstrated that the genetically obese mice had greater pulmonary inflammation at 8 dpi (Fig. 6E). LepRH<sup>H<sup>RH</sup>2/2</sup> mice exhibited higher levels of IL-10, MCP-1, and KC at 8 dpi.

In the dietary model of obesity, HFD mice had fewer BAL macrophages and Tregs. Therefore, the distribution of these cells in LepRH<sup>H<sup>H</sup>/fl/fl</sup> and LepRH<sup>H<sup>RH</sup>2/2</sup> mice was assessed at 4 and 8 dpi (Fig. 6F–H). Similar to HFD obese mice, LepRH<sup>H<sup>RH</sup>2/2</sup> mice had fewer BAL macrophages (Fig. 6F). Additionally, LepRH<sup>H<sup>RH</sup>2/2</sup> mice also had fewer CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells (data not shown), BAL Tregs, and activated BAL Tregs (Fig. 6G, 6H). Therefore, both HFD and LepRH<sup>H<sup>RH</sup>2/2</sup> obese mice exhibit similar differences in BAL macrophage and Treg numbers during pH1N1 infection in comparison with lean controls.

Obese LepRH<sup>H<sup>RH</sup>2/2</sup> mice exhibit distinct metabolic perturbations in lung tissue during pH1N1 infection

To enhance our analysis of the metabolic consequences of obesity during pH1N1 infection, the lungs of LepRH<sup>H<sup>H</sup>/fl/fl</sup> and LepRH<sup>H<sup>RH</sup>2/2</sup> mice were harvested at 0 and 8 dpi and processed for metabolic profiling. In Fig. 7A and 7B, the OPLS-DA plots demonstrate...
significant separation between lung samples from uninfected and infected LepRHfl/fl and LepRHH/H mice. LepRHH/H mice exhibited alterations in a number of lipid metabolites at both 0 and 8 dpi. Additionally, few metabolites differentially affected by obesity at 0 dpi were also different at 8 dpi, indicating that the recovered metabolic data at 8 dpi are specific to the consequences of obesity during pH1N1 infection, rather than being caused by obesity alone. Of interest, most metabolites different between LepRHfl/fl and LepRHH/H mice were lower in the lungs of LepRHH/H mice. A pathway enrichment analysis based on the 8 dpi lung metabolomes of LepRHfl/fl and LepRHH/H mice was constructed. Similar to pathways altered in the lungs of HFD mice, a number of the pathways altered in obese LepRHH/H mice were related to nucleotide or fatty acid metabolism as well as amino acid metabolism (Fig. 7C).

Metabolic profiling of urine can differentiate lean LepRHfl/fl mice and obese LepRHH/H at an early pH1N1 infection time point

We have previously demonstrated that 1H nuclear magnetic resonance–based metabolic profiling of urine can distinguish lean and obese mice infected with a sublethal dose of influenza A/Puerto Rico/8/1934 (H1N1) (26). Urinary profiling is both informative and readily translatable given that urine reflects a wide range of physiological and pathophysiological processes and is easily obtained in clinical settings (compared with tissues). We therefore sought to extend our metabolic analysis to include profiling of urine from uninfected and pH1N1-infected LepRHfl/fl and LepRHH/H mice. During the infection, we chose to profile urinary samples at 2 dpi. This time point occurs prior to drastic weight loss or obvious signs of sickness, allowing utilization of metabolic profiling for identifying biomarkers that may be used to predict or explain mechanisms of greater severity in LepRHH/H obese mice. The OPLS-DA plot in Fig. 8A demonstrates that UPLC-MS profiling was able to successfully distinguish urine from LepRHfl/fl and LepRHH/H mice at 0 and 2 dpi. A relatively large number of metabolites were detected at greater levels in the urine at 0 dpi in LepRHH/H mice (Fig. 8B). At 2 dpi, several unique metabolites were altered by infection in obese mice such as t-acetylcarnitine, phosphatidylcholine, and eight different amino acid–related metabolites (Fig. 8C). Lastly, pathway analysis of urinary metabolomes at 2 dpi revealed alterations in pathways related to fatty acid, amino acid, and histamine metabolism (Fig. 8D). Although it is known that obesity is characterized by alterations in fatty acid metabolism, we show that changes in fatty acid metabolic pathways (and several other pathways) occur systemically in the urine and locally in the lung during a pH1N1 infection.

Discussion

Obesity is a global epidemic, and the consequences of excess adiposity are diverse and continue to mount (43). Following the emergence of the 2009 pH1N1 strain, obesity was reported to be an independent risk factor for greater pH1N1 (4, 6, 17, 37, 38) and seasonal (44–46) influenza infection severity. In this study, we provide an in-depth analysis of the immunologic and metabolic complications associated with obesity during infection with the 2009 pH1N1 influenza. Previous studies in mice have shown that obesity alters lung wound healing and inflammatory responses during a pH1N1 infection (10), possibly due to hyperleptinemia (11). However, the complications of obesity are complex, and a number
of other factors could be affecting pH1N1 immunity such as hyperglycemia, hyperinsulinemia, oxidative stress, changes in the gut microbiome, and alterations in cellular and systemic metabolism (9). Therefore, we sought to combine immunological assessments with metabolomics to provide a more global characterization of the metabolic consequences of obesity on pH1N1 infection.

One of several mechanisms in which HFD-induced obesity may result in greater pH1N1 severity is the impact of consuming a HFD. Altering dietary composition, even without inducing nutritional deficiencies, can have systemic metabolic consequences (47–49) and can affect antiviral immunity (50). One inherent limitation with a diet-induced obesity model is that a HFD will always differ from a lean control diet (e.g., saturated fatty acid content, carbohydrate content), potentially confounding the implications of the impact of obesity. One goal of this investigation was to provide an in-depth analysis of the consequences of HFD-induced obesity in comparison with two different lean control groups to better assess dietary contributions. It has previously been demonstrated that LFDs high in sucrose can have metabolic consequences and can impact immune responses (51). Additionally, the CD differs in nearly every aspect from the purified LFDs and HFDs (yet it is still one of the most widely used control diets for obesity studies) (52). Several key differing components are amounts of phytoestrogens and dietary fiber. CDs are primarily derived from plant constituents and therefore contain phytoestrogens as well as dietary fiber (52). Phytoestrogens have been shown to affect metabolism and behavior. Furthermore, fiber can also impact metabolic health and modulate the gut microbiome (52, 53), and the purified LFD has no fiber (perhaps contributing to greater weight gain and a somewhat intermediate metabolic phenotype between CD mice and HFD mice as in Fig. 1). These differing characteristics of the diets likely explain some of the differential responses observed...
between the three dietary groups (especially between LFD and CD mice).

Currently, three studies have demonstrated that diet-induced obese mice exhibit greater mortality to a primary pH1N1 infection (10, 11, 15). Two of these studies used a CD for the lean control group and a 45 or 60% kcal HFD for obese mice (10, 11), and one study used a 60% kcal HFD compared with a nutrient-matched, purified 10% kcal LFD (15). Although we found that HFD mice were more susceptible compared with both lean groups, lean LFD mice exhibited greater mortality compared with lean CD mice. This demonstrates that modulating the diet alone, without inducing obesity, can increase pH1N1 severity, and it is possible there are synergistic effects of being obese and consuming a HFD. This outcome may have potential public health implications in that perhaps not only being obese increases risk for influenza severity, but the composition of the diet one chooses to eat could also impact severity as well.

Given the complications of diet-induced obesity models, a genetic model of obesity was also included in our analysis in an attempt to better assess the immunological consequences of excess adiposity independent of dietary effects. Obese male and female LepRH<sup>2<sup>2</sup></sup> mice were significantly more susceptible to pH1N1 mortality compared with lean control groups.

**FIGURE 8.** Metabolic profiling reveals alterations in distinct metabolic networks in urine of pH1N1-infected obese LepRH<sup>2<sup>2</sup></sup> mice. (A) OPLS-DA plot from urine samples from the same cohort of uninfected and 2 dpi mice. (B and C) Differentially altered metabolites in the urine of uninfected (B) and at 2 dpi (C) LepRH<sup>2<sup>2</sup></sup> mice compared with lean LepRH<sup>+/+</sup> mice (n = 3). (D) Pathway analysis generated in GeneGo of all pathways significantly different at 2 dpi between LepRH<sup>+/+</sup> and LepRH<sup>2<sup>2</sup></sup> mice. Next to each metabolite, *p < 0.05, **p < 0.005, or ***p < 0.0005. #, 1-nitro-7-glutathionyl-8-hydroxy-7,8-dihydronaphthalene; PPARγ, peroxisome proliferator-activated receptor γ.
leptin are a commonly used model of genetically induced obesity, in which lack of leptin satiety cues result in hyperphagia and obesity (54, 55). O’Brien et al. (10) have previously demonstrated that ob/ob obese mice were more susceptible to pH1N1 mortality. Although this is informative and helps to address the complications of dietary models discussed above, a global deficiency of leptin signaling can cause physiologic and immunologic complications, confounding the effects of obesity (55). Therefore, we used a genetic model of obesity in which disruption of leptin signaling is isolated to hypothalamic neurons (although there is some evidence of Nkx2.1 expression in the lungs, esophagus, and during development) (32). This genetic model of obesity established that although diet may synergize with obesity to impact pH1N1 immunity, obesity alone is sufficient to increase pH1N1 mortality. Additionally, this study highlights the importance of careful consideration in choosing a mouse model of obesity (genetic- versus diet-induced) and in selecting proper dietary controls for HFD-induced obesity studies.

Both diet- and genetic-induced obese mice exhibited greater lung damage during the pH1N1 infection. This excess lung pathology has been demonstrated previously in obese mice, and it is likely due to impaired wound healing in the lung (10). A number of potential mechanisms may be responsible for enhanced lung damage in obese mice. Tregs are critical regulators of immunopathology and have been shown to limit inflammatory responses to respiratory syncytial virus and some influenza infection models in mice (39–41). We have previously demonstrated that obese mice have fewer Tregs in the lung airways during a secondary pH1N1 challenge, and Tregs isolated from obese mice exhibited impaired suppressive capacity compared with Tregs isolated from lean mice (14). Therefore, we also investigated the distribution of Tregs in the lung, lung airways, and mLN during a primary pH1N1 infection. At 8 dpi, HFD- and genetic-induced obese mice had fewer BAL Tregs and fewer activated BAL Tregs. It is currently unclear why obese mice have fewer BAL Tregs during influenza infection, but it is well established that obesity results in a deficiency of Tregs in metabolic tissues such as the liver and white adipose tissue (8, 9). Obesity results in greater lung damage during both primary and secondary pH1N1 infections, and therefore uncovering how obesity modulates BAL Treg responses is likely important for understanding the complex mechanisms of influenza severity caused by an obese state.

Additionally, fewer F4/80+ macrophages were detected in the lung airways of obese mice at 4 dpi in HFD mice and at 8 dpi in LepRH+/− mice. NK cell numbers were also slightly lower in HFD mice compared with CD mice, but this did not reach statistical significance (p = 0.07). Macrophages fulfill a diverse number of processes in infection recovery such as propagating inflammatory responses or promoting wound healing (16, 56). For example, alternatively activated macrophages are prototypically anti-inflammatory and facilitate wound healing (16). Although we did not phenotype BAL macrophages, perhaps obese mice had fewer BAL alternatively activated macrophages during infection, contributing to greater immunopathology. Future investigations are necessary to assess how obesity modulates macrophage polarization and function in the lung during pH1N1 infection.

Obesity is inherently a metabolic disease. Therefore, we hypothesized that metabolic profiling can be used to enhance our current understanding of the consequences of obesity on influenza infection. A partial least squares discriminant analysis of the broad-spectrum metabolomics data successfully distinguished lung samples on the basis of both obesity and infection status. In lung tissue from uninfected and infected HFD mice, several lipid species related to fatty acid, cholesterol, or phospholipid metabolism were altered compared with CD mice. Of interest, the number of lipid metabolites altered in HFD lungs nearly tripled by 8 dpi, with some lipid metabolites such as 3-oxododecanoic acid and 4-hydroxyisovaleric acid detected at nearly 10-fold greater concentrations. Obesity is associated with greater levels of circulating unesterified fatty acids and TAG (8), but why these lipid metabolites are altered in the lungs of obese mice at 8 dpi (rather than in uninfected mice or at 4 dpi) is unknown. Perhaps the complex milieu of excess inflammatory cytokines and metabolic hormones in the lungs of obese mice impacts cellular metabolism, ultimately impeding proper lung healing and recovery.

Metabolic profiling of lung tissue revealed several interesting findings. p-Cresol sulfate was detected at a 5-fold greater concentration in uninfected HFD obese mice compared with CD mice. By 4 dpi, p-cresol sulfate was detected at a 55-fold greater level in the lungs of HFD obese mice and remained elevated at 8 dpi, with HFD mice showing >10-fold p-cresol sulfate levels. p-Cresol sulfate is a metabolite derived from secondary metabolism of p-cresol by gut microbiota, typically reported to be found in the urine (57). p-Cresol sulfate accumulates during conditions of kidney failure, and some evidence indicates it may be toxic (57, 58) and can impact immune cell function (59). Although we did not measure urinary p-cresol sulfate in HFD mice, perhaps elevated levels of this metabolite may reflect impaired kidney function. Additionally, given that p-cresol sulfate is a microbial-derived metabolite, it is also possible that influenza differentially alters gut microbiota function in obese mice, ultimately impacting antiviral immune responses. It has previously been demonstrated that altering the gut microbiome can affect influenza infection immunity in mice (60). Of interest, LFD mice also exhibited greater concentrations of lung p-cresol sulfate compared with CD mice, suggesting that perhaps elevated p-cresol sulfate levels are due to dietary–microbiome interactions. There is little known regarding p-cresol sulfate in the lung or during the context of an infection, but the strikingly elevated levels detected in the lungs of HFD mice merit further investigation.

A number of other metabolites notably elevated in the lungs of HFD mice include glutamyl-proline (increased in HFD mice at 4 and 8 dpi), tetrahydrocortisol (4 dpi), 3-hydroxybutyric acid (8 dpi), and the numerous acylcarnitine species elevated at 8 dpi in HFD lungs. Chronic overnutrition is thought to increase cytosolic lipid content in insulin-sensitive tissues (such as liver and skeletal muscle), which can ultimately impair insulin signaling (61). Perhaps the elevated levels of acylcarnitines reflect these processes in the lung. Furthermore, accumulation of acylcarnitines may indicate impaired fatty acid oxidation in lung cells (61). Taken together, the diverse changes in the lung metabolome of HFD mice during pH1N1 may reflect pathological processes contributing to greater infection severity.

LepRH+/− mice also exhibited alterations in an array of lung metabolites prior to and during the pH1N1 infection. One notable difference between HFD obese mice and LepRH+/− obese mice was that most differentially altered metabolites in HFD mice were elevated compared with CD mice, whereas most metabolites in LepRH+/− mice were detected at a lower concentration compared with LepRH+/− mice. Furthermore, relatively few metabolites were similarly altered in both obesity models. At 8 dpi, only arachidonic acid and hydroxyvaleryl-carnitine were elevated in the lungs of both HFD and LepRH+/− mice compared with lean controls. Of interest, arachidonic acid is a substrate for eicosanoid metabolism, and arachidonic acid metabolites (such as leukotrienes and PGs) can impact inflammatory responses (62). The fact that the lung metabolomes in HFD and LepRH+/− mice differ greatly highlights the impact of the diet in this model. Future studies are required to better understand the synergistic effects of a HFD and excess adiposity, as well as how these factors interact to modulate the lung metabolome and affect pH1N1 immunity.
We also performed metabolic profiling on urine samples from LepR^+/+ and LepR^−/− mice at 2 dpi. Similar to changes in lung metabolites, alterations in urinary metabolites related to fatty acid, nucleotide, and amino acid metabolism were measured. The fact that metabolic profiling can distinguish LepR^−/− mice from lean control mice as early as 2 dpi (prior to any obvious signs of illness or drastic weight loss) introduces the possibility of utilizing metabolic profiling to complement current prognostic or diagnostic approaches in the future. Additionally, the urinary metabolome may provide insight into the mechanisms in which obesity increases pH1N1 mortality. For example, taurine was elevated in the urine of obese LepR^−/− mice at 2 dpi. Detection of taurine has been reported to be increased under conditions of oxidative stress (63). The fold change of taurine increased from slightly greater in uninfected urine of LepR^−/− mice to 4-fold greater by 2 dpi. Therefore, this may indicate that influenza infection induces greater oxidative stress in obese mice.

Taken together, we have provided a comprehensive analysis of both the immunologic and metabolic consequences of obesity during pH1N1 infection. It is likely that the distinct metabolic perturbations detected in the lungs of obese mice are both reflective and instructive of pathologic changes occurring in the lung following infection. Additionally, we were able to document distinct changes in the urine of infected mice before overt signs of illness, demonstrating the strength of this approach. Future studies are required to further integrate these metabolomic data with immunologic responses to identify novel mechanisms for greater pH1N1 severity in obese mice.

Disclosures
The authors have no financial conflicts of interest.

References