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Diet-Induced Obese Mice Exhibit Altered Heterologous Immunity during a Secondary 2009 Pandemic H1N1 Infection

J. Justin Milner,* Patricia A. Sheridan,* Erik A. Karlsson,† Stacey Schultz-Cherry,† Qing Shi,* and Melinda A. Beck*

During the 2009 pandemic H1N1 influenza A virus (pH1N1) outbreak, obese individuals were at greater risk for morbidity and mortality from pandemic infection. However, the mechanisms contributing to greater infection severity in obese individuals remain unclear. Although most individuals lacked pre-existing, neutralizing Ab protection to the novel pH1N1 virus, heterologous defenses conferred from exposure to circulating strains or vaccination have been shown to impart protection against pH1N1 infection in humans and mice. Because obese humans and mice have impaired memory T cell and Ab responses following influenza vaccination or infection, we investigated the impact of obesity on heterologous protection from pH1N1 infection using a mouse model of diet-induced obesity. Lean and obese mice were infected with influenza A/Puerto Rico/8/34 (PR8) and 5 wk later challenged with a lethal dose of heterologous pH1N1. Cross-neutralizing Ab protection was absent in this model, but obese mice exhibited a significantly lower level of nonneutralizing, cross-reactive pH1N1 nucleoprotein Abs following the primary PR8 infection. Further, obese mice had elevated viral titers, greater lung inflammation and lung damage, and more cytotoxic memory CD8+ T cells in the lung airways. Although obese mice had more regulatory T cells (Tregs) in the lung airways than did lean controls during the pH1N1 challenge, Tregs isolated from obese mice were 40% less suppressive than Tregs isolated from lean mice. In sum, excessive inflammatory responses to pH1N1 infection, potentially owing to greater viral burden and impaired Treg function, may be a novel mechanism by which obesity contributes to greater pH1N1 severity.

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Abbreviations used in this article: BAL, bronchoalveolar lavage; dpi, days postinfection; GzB, granzyme B; HAI, hemagglutination inhibition; MDCK, Madin–Darby canine kidney (cell); MFI, median fluorescence intensity; mLN, mediastinal lymph node; MOL, multiplicity of infection; NP, nucleoprotein (influenza); pH1N1, pandemic H1N1 2009 influenza A virus; PR8, influenza A/Puerto Rico/8/34; RDE, receptor destroying enzyme; TCID50, 50% tissue culture infective dose; Teff, effector T cell; Treg, regulatory T cell.

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aspects of the immune response and increases susceptibility for a variety of pathogens, including influenza virus (37). Although recent investigations in mice and humans have begun to elucidate potential mechanisms by which obesity impairs antiviral immunity to influenza infection, the specific factors contributing to the increased severity observed in the obese population remain unclear (37). We have previously demonstrated that obese mice and humans exhibit impaired memory CD8+ T cell responsiveness to influenza stimulation (38, 39). Further, obese mice display impaired responsiveness to influenza vaccination (40), and obese humans are unable to maintain long-term influenza Ab levels following vaccination (39). Therefore, given the protective nature of cross-reactive Ab and T cell responses, we hypothesized that obesity impaired heterologous immunity induced by previous influenza exposure, resulting in greater pH1N1 infection severity.

In this study, we used a model in which lean and obese mice were initially infected with a sublethal dose of influenza A/Puerto Rico/8/34 (PR8). After 5 wk, mice were challenged with a lethal dose of heterologous pandemic A/Cal/04/09 (H1N1, pH1N1). Similar to results shown previously in lean, chow-fed mice (21), we found that priming with PR8 effectively prevented mortality from pH1N1 infection in both lean and obese mice in the absence of cross-reactive neutralizing Abs. However, obese mice exhibited a lower level of cross-reactive pH1N1 NP Abs 5 wk after the PR8 infection, and a lower proportion of obese mice generated pH1N1 hemagglutination inhibition (HAI) Abs following the secondary challenge. Consequently, obese mice had higher lung viral titers, more lung disease, and more cytokotoxic memory CD8+ T cells in lung airways during the pH1N1 infection. Given the excessive inflammation, infiltration, damage, and cytokotoxic CD8+ T cell responses in the lungs of obese mice during the lethal pH1N1 challenge, we investigated the impact of obesity on regulatory T cells (Tregs) as a potential mechanism for the inability to control the antiviral responses in the lung. Unexpectedly, obese mice, compared with lean mice, had nearly twice as many Tregs in the lung airways during the heterologous secondary pH1N1 infection. However, ex vivo analysis of Treg function revealed that Tregs isolated from obese mice were significantly less suppressive than those isolated from lean mice. Therefore, an excessive inflammatory response in the lungs, potentially owing to a combination of elevated viral titers and impaired Treg function, may be a mechanism by which obesity enhances pH1N1 infection severity.

Materials and Methods

Mice and diets

Weanling, male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and fed a low-fat diet with 10% kcal fat (D12450; Research Diets). In addition to inducing weight gain, high-fat diets for 15–18 wk, as described below. Mice were housed in isolation cages (D12451; Research Diets). In addition to inducing weight gain, high-fat diets are considered proinflammatory (41). Mice were maintained on the diets for 15–18 wk, as described below. Mice were housed in isolation cubicles at the University of North Carolina Animal Facility (fully accredited by the American Association for Accreditation of Laboratory Animal Care). All experimental procedures involving mice were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Influenza virus and infection in mice

H1N1, PR8—obtained from American Type Culture Collection (Manassas, VA)—was used for primary infections. H1N1, pH1N1 was obtained from BEI Resources (Manassas, VA) and was used for secondary infections. Both PR8 and pH1N1 were propagated in the allantoic cavities of 10- to 12-d-old embryonated chicken eggs. At 72 h postinfection, allantoic fluid from eggs was harvested and clarified by centrifugation at 5000 × g for 10 min at 4˚C, aliquoted, and stored at −80°C. The stock viral titers of PR8 and pH1N1 were determined by a modified 50% tissue culture infective dose (TCID50) in Madin–Darby canine kidney (MDCK) cells, using hemagglutination as an endpoint (42), and evaluated by the method of Reed and Muench (43). For influenza inoculations, mice were lightly anesthetized by isoflurane inhalation and inoculated via noninvasive oral aspiration (44, 45) with 0.05 ml viral inoculum diluted in PBS. For rechallenge studies, mice were maintained on the designated low-fat or high-fat diet for 15 wk and then infected with 1.4 TCID50 PR8 for a primary infection. Mice were monitored and weighed daily for 14 d postinfection (dpi) following the primary PR8 infection. At 5 wk after the primary PR8 infection, a similar period as that used by others (27, 30, 38, 46), obese mice were rechallenged with 5 × 103 TCID50 pH1N1, a previously determined lethal dose (~10 LD50). Following the secondary pH1N1 infection, mice were weighed daily. For isolation of Tregs from mouse spleenocytes or harvesting of sera during the primary PR8 infection, mice were maintained on a high-fat or low-fat diet for 18 wk.

Quantitation of viral titers

Viral titers in lung tissue were determined by a modified TCID50, using hemagglutination as an endpoint, as previously described (47). Briefly, the lungs of 35 from lavaged mice were harvested and frozen in liquid nitrogen for subsequent processing. The supernatant from each homogenized lung was collected, serially diluted, and added to 80% confluent MDCK cells in triplicates of six in 96-well plates. TCID50 was determined using the Reed and Muench method (43), and values were normalized to lung tissue weight (38).

HAI and microneutralization assays

Sera were collected from individual mice, at days 0 (5 wk after the primary PR8 infection, naive to pH1N1), 5, 8, and 14 following the secondary pH1N1 challenge, and HAI titers were determined. Briefly, sera were treated with receptor destroying enzyme (RDE; Denka Seiken, Tokyo, Japan) overnight, followed by inactivation at 56˚C for 1 h, and a final dilution to 1:10 with PBS. RDE-treated sera were then incubated with pH1N1, PR8, or influenza A/Victoria/361/2011 (negative control) for 15 min at room temperature (primary PR8 infection sera were incubated with PR8 alone). After a 1-h incubation at 4˚C with either 0.5% turkey RBC (PR8, pH1N1) or 0.5% chicken RBC (PR8, A/Victoria/361/2011), HAI titer was determined by the reciprocal of the highest dilution of serum to completely inhibit hemagglutination. Positive and negative controls, as well as back titrations of virus, were included on each individual plate.

Microneutralization assays were performed on sera from PR8-infected mice at 35 dpi, as previously described (48). Briefly, 100 TCID50 of either PR8 or CA/09 was added to 2-fold dilutions of RDE-treated sera, and serum–virus mixtures were incubated at 37˚C, 5% CO2, for 1 h. Following incubation, 3 × 105 MDCK cells were added to each well, and plates were incubated overnight at 37˚C, 5% CO2. Plates were then fixed with 80% acetone and blocked for 2 h at room temperature, and an ELISA was performed using a mouse anti-influenza A NP mAb mixture (BEI Resources, followed by peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA). ELISA plates were developed for 10 min, using Substrate Reagent (R&D Systems, Minneapolis, MN), then stopped using a 2N H2SO4 solution, and absorbance of each well was read at 450 nm. Wells were considered positive for microneutralization at an OD ≤ 50% of the MDCK cells being infected.

Influenza NP ELISA

Anti-NP Abs in the sera of lean and obese mice 35 d after the primary PR8 infection were measured by ELISA. Briefly, 8 μg/5 μl of purified pH1N1 NP (A/California/06/2009; Immune Technology, New York, NY) was coated on ELISA plates (BD Falcon, San Jose, CA) overnight at 4˚C in coating buffer. Influenza A/Cal/04/2009 NP was not commercially available, so purified NP from the A/Cal/06/2009 pandemic strain was used. Subsequently, ELISA plates were blocked with 1% BSA for 1 h at 37˚C, washed, and then incubated with mouse sera (1:400 dilution) overnight. Following the overnight incubation, plates were washed and incubated with HRP-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA) Ab (1:2000 dilution) for 1 h at 37˚C. After washing, the assay was developed with the TMB Substrate Kit (Thermo Scientific, Rockford, IL) per the manufacturer’s instructions. OD was measured at 405 nm, and the OD of uninfected control sera was subtracted from the 35 dpi experimental samples.

Lung histopathology

At 5 dpi, the left lobe of the lung was harvested from lean and obese mice, inflated with 4% paraformaldehyde and maintained in 4% paraformaldehyde for 48 h, after which samples were transferred to 70% ethanol. Tissues were paraffin embedded, and three 5-μm (separated by 100 μm) sections (per lung sample) were processed for H&E staining by the University of
To recover bronchoalveolar lavage (BAL) fluid, the tracheas of killed mice were exposed and cannulated with a 22-gauge angiocath, and the lungs were then lavaged with a series of four washes with unsupplemented HBSS, totaling 75 ml (0.75-ml and three 1-ml washes). BAL fluid supernatant was collected from the initial 0.75-ml lavage and was subsequently used for total protein and albumin detection. The cells from the series of BAL washes for each mouse were combined for subsequent flow cytometry analysis. BAL fluid was harvested at 5, 8, and 14 d following the secondary pH1N1 infection. BAL supernatants from 5 and 8 dpi were diluted 1:10, and total protein was measured (BCA Assay Kit; Sigma-Aldrich, St. Louis, MO). BAL supernatants were diluted 15,000-fold prior to measuring albumin levels, per the manufacturer’s instructions for the Mouse Albumin ELISA Kit (GenWay Biotechn, San Diego, CA).

**Quantitation of lung cytokine gene expression**

Lung tissue samples were collected at day 0 (5 wk after the primary PR8 infection, naive to pH1N1) and 5, 8, and 14 d following the secondary pH1N1 infection. Total RNA was isolated using the TRIzol method (Invitrogen), and reverse transcription was performed with use of the SuperScript II First-Strand Synthesis Kit (Invitrogen), using oligo (dT) primers. Expression levels of cytokines and chemokines were quantified using quantitative RT-PCR, as previously described (42).

**Flow cytometry**

Splenocytes and cells from draining mediastinal lymph nodes (mLNs) were isolated as previously described (38). For analysis of surface proteins of T cell populations, single-cell suspensions were simultaneously incubated with cell block anti-CD16/CD32 and stained in PBS (1% FBS) with the following mAbs: CD3 (APC/Cy7), CD62L (Brilliant Violet 421), and CD8 (PerCP) from BioLegend (San Diego, CA); CD4 (FITC, PE-Cy7), CD25 (PE-Cy7), CCR7 (APC), CD44 (PE-Cy7), and CD62L (E450) from eBioscience (San Diego, CA). MHC class I tetrameric complexes (PE) specific for the H-2Db–restricted epitope of the NP (NP, 3–16; E450) from eBioscience and the irrelevant lymphocytic choriomeningitis virus tetramer D0G3–34 (PE) was used as a negative control (National Institutes of Health Tetramer Core Facility, Atlanta, GA). For intracellular cytokine and transcription factor staining, cells were fixed and permeabilized with the Foxp3 Transcription Factor Staining Buffer Set (eBioscience) per the manufacturer’s instructions. Subsequently, cells were stained with Foxp3 (APC; eBioscience) for identification of Tregs. For detection of irrelevant lymphocytic choriomeningitis virus tetramer D0G3–34 (PE) or the Foxp3 Transcription Factor Staining Buffer Set (eBioscience) per the manufacturer’s instructions. Subsequently, cells were stained with Foxp3 (APC; eBioscience) for identification of Tregs. For detection of irrelevant lymphocytic choriomeningitis virus tetramer D0G3–34 (PE) or the Foxp3 Transcription Factor Staining Buffer Set (eBioscience). 

**Suppression assay**

Treg suppression assays were performed similarly to those done by De Rosa et al. (49). Tregs were isolated from pooled splenocytes of three naive lean or three naive obese mice. CD4+CD25+ Tregs and CD4+CD25− effector T cells (Teff) were isolated using the Dynabeads FlowComp Mouse CD4+CD25+ Treg Cells Kit (Invitrogen) and stimulated with Dynabeads mouse anti-CD3/28 (0.5 bead per T eff; 5 × 10^5 T eff per well). The Tregs (≥95% CD25−) were cultured at a ratio of 1:2 in round-bottom 96-well plates with complete RPMI 1640 medium. Only T eff from lean mice were used in the assay to eliminate concern for a discrepancy in obese mouse T eff proliferation. Cells were stimulated for 72 h, and cells were pulsed with [3H]thymidine (PerkinElmer, Boston, MA) for the last 16 h of culture. Cells were harvested onto glass fiber filter paper (Brandel, Gaithersburg, MD), and radioactivity was measured using a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland).

**Statistical analysis**

JMP Statistical Software (SAS Institute, Cary, NC) was used for all statistical analyses. Statistical significance for parametric data was evaluated using the two-tailed unpaired Student’s t test to compare dietary groups. For nonparametric data, the Wilcoxon signed-rank test was used. Finally, for the percentage of mice with HAI titers at each dpi, the two-tailed Fisher exact test was used. Differences between means were considered significantly different at p < 0.05.

**Results**

**Heterologous immune defenses prevent mortality from a lethal pH1N1 challenge in lean and obese mice**

To investigate the impact of obesity on heterologous immunity to a lethal pH1N1 infection, we used a diet-induced obese mouse model. To induce obesity, weaning, male C57BL/6J mice were fed a high-fat (45% kcal from fat) diet or a low-fat (10% kcal from fat) control diet for 15 wk (Fig. 1A). The OpenSource Diets are composed of purified constituents and are matched in every aspect except for fat and carbohydrate content. Mice fed a high-fat diet rapidly gained more weight than control mice and after 15 wk on the diet weighed ∼33% more (Fig. 1B). Mice were maintained on the respective high-fat or low-fat diet throughout the course of the experiment, including both primary and secondary infections.

To test whether obese mice had compromised heterologous defenses, lean and obese mice were initially infected with a sub-lethal dose of PR8 virus after the 15 wk of dietary exposure (Fig. 1A). As expected, obese mice lost significantly more weight (5.10 ± 0.40 g) than did lean mice (3.54 ± 0.31 g) following the primary PR8 infection, but when normalized to preinfection body weight, no differences in percent weight loss were noted between lean and obese mice (Fig. 1C). PR8-infected mice were maintained on their respective diets for 5 wk to allow the mice sufficient time to recover. After 5 wk, having regained weight equivalent to initial weight status prior to the PR8 infection (data not shown), mice were infected with a lethal dose of pH1N1 virus. Fully 100% of naive (unprimed) lean and obese mice rapidly succumbed to the lethal pH1N1 infection at 6 dpi (Fig. 1D). However, of the mice previously infected with PR8, 100% of lean mice and 95% of obese mice survived the pH1N1 challenge. Contrary to the initial primary infection, both lean and obese mice began to lose weight early at 2 dpi following the secondary pH1N1 infection, with obese mice losing nearly twice as much weight as lean mice over the course of the infection. However, once normalized to body weight, weight loss was not significantly different between the two groups (Fig. 1E).

**Diet-induced obesity impairs primary and secondary influenza Ab responses**

We assayed sera for HAI Abs 5 wk following the primary PR8 infection in lean and obese mice to confirm that the pH1N1 virus was heterologously distinct in our hands. We did not detect HAI Abs to pH1N1 in sera from lean or obese mice previously infected with PR8 (Fig. 2A). Further, microneutralization assays confirmed that the PR8 infection did not induce cross-neutralizing protection to pH1N1 virus (Fig. 2B). Similarly, Guo et al. (21) did not detect cross-neutralizing Abs to pH1N1 in sera from PR8-infected mice. Therefore, reflective of what happened in the human population during the pH1N1 outbreak, PR8-infected mice lacked pH1N1 cross-reactive neutralizing Abs.
As expected, 5 wk after PR8 infection, lean mice displayed elevated HAI and microneutralization titers to PR8, but not pH1N1 (Fig. 2A, 2B). Surprisingly, none of the obese mice had detectable HAI Abs to PR8, and obese mice had nearly a 4-fold lower PR8 microneutralization titer. Although PR8 HAI Abs are not critical modulators of secondary infection outcome in this model, the striking deficiency in PR8 HAI Abs prompted us to measure PR8 Ab levels during the primary PR8 infection to determine if the reduced Ab levels at 35 dpi were a result of decreased Ab generation. At 7 dpi following the primary PR8 infection, obese mice had a lower mean PR8 HAI titer, and a lower percentage of obese mice (p = 0.06) exhibited detectable PR8 HAI Abs (Fig. 2C, 2D). However, by 9 dpi and through 14 dpi, obese mice had levels of HAI Abs similar to those in lean mice. Therefore, obese mice exhibited delayed Ab generation during the primary infection but were able to compensate and eventually exhibited levels similar to those in lean mice. However, by 35 dpi, obese mice did not have any detectable PR8 HAI Abs (Fig. 2C, 2D), indicating that obesity also impairs Ab maintenance. Kim et al. (40) demonstrated that obese mice have impaired responsiveness to influenza vaccination, but obesity-induced impairments in Ab responses following infection in mice have not previously been shown. Of interest, we have similarly demonstrated that obese humans have an impaired ability to maintain long-term levels of influenza Abs (39).

Because of the striking deficiency of PR8 HAI Abs in the obese mice at 35 dpi, we also assessed pH1N1 HAI Ab levels following the secondary challenge. Previous studies in mice have demonstrated that a primary heterologous influenza infection can lead to an accelerated production of pH1N1-neutralizing Abs during a secondary pH1N1 infection (25, 30), which could potentially
affect infection outcome. We did not detect HAI Abs at 5 dpi in lean or obese mice; however, 72.7% of lean mice had HAI Abs at 8 dpi. By 14 dpi, 100% of lean mice had HAI Abs (Fig. 3A, 3B). In contrast to lean mice, 41.7% of obese mice generated detectable serum HAI Abs at 8 dpi, and only 50% of obese mice had pH1N1 HAI Abs at 14 dpi (Fig. 3A, 3B). Therefore, obese mice displayed altered Ab responses during both primary and secondary infections. Although PR8 HAI Abs offer negligible protection during the secondary infection, we measured PR8 HAI Abs to determine if the impairments in Ab production during the secondary infection were specific to pH1N1 or a more global defect. The differences between lean and obese mice were striking, as 75% (day 5), 72.7% (day 8), and 83.3% (day 14) of lean mice had detectable levels of HAI Abs to PR8 during the secondary pH1N1 infection, compared with 8.3% (day 5), 16.7% (day 8), and 33.3% (day 14) of obese mice (Fig. 3C, 3D). As a negative control for all HAI titers from sera obtained during the secondary infection, influenza A/Victoria/361/2011 (H3N2) was assayed in addition to pH1N1 and PR8. Taken together, obese mice exhibited delayed or impaired Ab generation during primary and secondary influenza infections, respectively, and impaired Ab maintenance (35 d following the primary PR8 infection).

Although PR8 Abs do not neutralize pH1N1 virus infectivity (see Fig. 2B), NP-specific Abs generated during a heterologous primary influenza infection or through vaccination have been shown to control virus replication during influenza infections (29, 50, 51). Therefore, we next assayed the level of anti-pH1N1 NP Abs in lean and obese mice at 35 dpi. Of note, we detected that obese mice had a significantly lower level of serum anti-pH1N1 NP Abs (Fig. 3E). Because NP Abs can regulate viral burden (29), we next measured lung viral titers in lean and obese mice to determine if lower anti-NP Ab levels potentially affected influenza viral titers during the secondary pH1N1 infection. At 5 dpi, obese mice, compared with lean mice, had >10-fold higher mean virus titer (p = 0.05) in the lungs (Fig. 3F). Although viral replication is controlled through several cell types and effector responses, it is plausible that reduced NP Ab levels resulted in greater viral burden in the lungs of obese mice.

Obese mice develop greater lung airway inflammation following a secondary heterologous pH1N1 infection

Given that obese mice exhibited elevated viral titers at 5 dpi, we next assessed how greater viral burden had an impact on lung inflammation and disease. Enumeration of BAL cells following the secondary infection revealed that obese mice had nearly twice as many cells in the lung airways at 5 dpi (Fig. 4A). Conversely, in the draining mLNs, lean mice had approximately twice as many immune cells as obese mice at 5 dpi (Fig. 4B). Despite the lack of differences in inflammatory cytokine expression (Table I), obese mice had elevated lung pathology scores (Fig. 4C) and a greater level of BAL fluid protein at 5 dpi (Fig. 4D). To determine if the elevated level of immune cell infiltration and inflammation in the lungs resulted in damage to the integrity of the respiratory epithelium in obese mice, we assayed albumin levels in the lung airways. Consistent with heightened infiltrate and disease at 5 dpi, we detected greater levels of BAL albumin at 5 dpi in obese mice (Fig. 4E).

Obese mice have a heightened memory CD8+ T cell response in the lung airways following a lethal secondary pH1N1 infection

The protective role of memory T cells during secondary heterologous influenza infections is well established (52, 53). Further, several recent investigations have demonstrated that exposure to seasonal influenza strains or vaccination can elicit memory T cells that are able to recognize viral epitopes of pH1N1, conferring cross-protection (15, 25). Both CD4+ and CD8+ memory T cells have been shown to be important in limiting disease severity in several models of pH1N1 rechallenge studies (21, 25, 27).

To determine if obesity influenced the distribution and activation of cross-reactive T cells, BAL cells from mice previously infected with PR8 5 wk earlier and then challenged with pH1N1 were stimulated with heat-inactivated pH1N1 virus, pH1N1-pulsed APCs, or the polyclonal mitogen Con A at days 5, 8, and 14 following the secondary pH1N1 infection. Fig. 5A demonstrates that all three methods of stimulation induced a robust IFN-γ response...
in cross-reactive CD4+ T cells at 5 dpi. As expected, the addition of influenza-pulsed APCs in culture with BAL cells resulted in an elevated IFN-γ response, compared with BAL cells incubated with heat-inactivated virus alone. Obesity did not impair IFN-γ or GzB production by memory CD4+ T cells in response to the stimuli (Fig. 5B, 5C). No differences were observed in CD4+ T cell IFN-γ median fluorescence intensity (MFI) or GzB MFI (data not shown).

Next, we investigated potential differences in CD4+ effector memory T cells between lean and obese mice, as we have previously demonstrated that obese mice have an impaired CD8+ effector memory T cell response (38). Effector memory T cells were distinguished by lack of expression of the cell adhesion molecule CD62L, which is inherently absent or low on effector memory T cells (54). Because we detected differences in viral titers and lung pathology at 5 dpi, we focused on effector memory T cell populations specifically on this day. However, we did not detect any differences in IFN-γ or GzB production of CD4+CD62L- effector memory T cells between lean and obese mice (Fig. 5D, 5E). This finding suggests, at least partially, that cross-reactive CD4+ T cell cytotoxic responses are intact in obese mice.

We also determined how obesity affected the cross-reactive CD8+ T cell response under the same conditions described for CD4+ T cells. Approximately, 4–5% of CD8+ T cells from lean and obese mice produced IFN-γ following stimulation with influenza-pulsed APCs at 5 dpi (Fig. 6A). After day 5, the magnitude of the CD8+ T cell response declined in both lean and obese mice (Fig. 6B, 6C). Interestingly, obese mice had a strikingly elevated number of CD8+IFN-γ+ and CD8+GzB+ T cells at 5 dpi (Fig. 6D, 6E). Further analysis of the CD8+ memory T cell population revealed that obese mice had an elevated number of CD8+CD62L- IFN-γ+ T cells and a significantly greater number of CD8+CD62L- GzB+ effector memory T cells at 5 dpi as well (Fig. 6F, 6G). There were no differences in CD8+ IFN-γ MFI or GzB MFI (data not shown).

We used whole virus in BAL cell stimulations, and thus we were assessing the total polyclonal response of the CD4+ and CD8+ memory T cell pool. Because we detected significant differences in the number of GzB-producing effector memory CD8+ T cells in obese mice, we next examined GzB production by effector memory T cells specific for a specified viral epitope. As NP from PR8 shares 91% similarity with the pH1N1 NP (30), we used the influenza tetramer DbNP366–74, allowing for detection of T cells specific for the PR8 NP366–74 epitope during the secondary pH1N1 infection. Fig. 6F demonstrates that obese mice had a greater number of NP366–74–specific T cells producing GzB following stimulation. Further, obese mice had a significantly greater number of NP366–74–specific CD8+CD62L- T cells, and a significantly greater proportion of these cells produced GzB (Fig. 6F). The elevated cross-reactive CD8+ T cell response could be attributed to the greater viral burden detected in the lungs of obese mice at 5 dpi. It is likely that greater viral load induced excessive recruitment of CD8+ T cells to the lungs, and perhaps the elevated CD8+ T cell response was required to control the excess viral burden in the absence of proper Ab defenses.
Obese mice exhibit a greater inflammatory and pathological response in the lungs following a secondary heterologous pH1N1 challenge. (A and B) Enumeration of BAL cells (A) and mLN cells (B) at 5, 8, and 14 dpi following the secondary pH1N1 challenge; n = 9–12. Each data point represents an individual animal. (C) Representative H&E-stained lung histology slides at ×40 and ×100 magnification, and pathology score at 5 dpi, n = 3–4. (D) Total protein in BAL fluid at 5 and 8 dpi; n = 5–6. (E) Albumin levels in BAL fluid at 5 and 8 dpi; n = 5–6. Bars represent mean ± SEM; *p < 0.05, **p < 0.005 compared with lean mice.

Obese mice have a greater number of CD4+CD25+Foxp3+ Tregs in the lung airways at 5 dpi
Numerous factors could contribute to the increased lung inflammation, lung infiltration, lung damage, and memory CD8+ T cell responses in obese mice. Enhanced inflammatory responses in the lungs between 1 and 5 dpi or altered expression of lung-trafficking molecules on immune cells could result in the observed differences in infiltration in the lung at 5 dpi. However, we did not detect any differences in the percentage of mLN CD8+ T cells expressing the trafficking molecules CD44, CD62L, and CCR7 at 5 dpi, nor did we find any differences in mLN or lung chemokine expression at 0 or 5 dpi (mLN data not shown).

We observed greater infiltration, increased lung damage, and heightened CD8+ T cell responses in the lungs of obese mice, and

Table I. Similar levels of lung cytokine and chemokine expression during a secondary heterologous pH1N1 challenge in lean and obese mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Dietary Group</th>
<th>Day 0</th>
<th>Day 5</th>
<th>Day 8</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Lean</td>
<td>1.00 ± 0.11</td>
<td>1.92 ± 0.44</td>
<td>0.89 ± 0.12</td>
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<tr>
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<td>0.69 ± 0.10</td>
<td>0.59 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
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<td>2.64 ± 0.57</td>
<td>0.59 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.53 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>IFN-γ</td>
<td>Lean</td>
<td>1.00 ± 0.32</td>
<td>6.33 ± 0.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.41 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.96 ± 0.10</td>
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<td>Obese</td>
<td>0.71 ± 0.15</td>
<td>6.58 ± 1.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.01 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.81 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>IL-10</td>
<td>Lean</td>
<td>1.00 ± 0.18</td>
<td>6.62 ± 0.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.84 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.94 ± 0.05</td>
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<td></td>
<td>Obese</td>
<td>1.11 ± 0.34</td>
<td>8.31 ± 5.09</td>
<td>0.49 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.14 ± 0.03</td>
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<td>RANTES</td>
<td>Lean</td>
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<td>0.69 ± 0.08</td>
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<td>0.48 ± 0.08</td>
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<td>0.76 ± 0.09</td>
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<td>MCP-1</td>
<td>Lean</td>
<td>1.00 ± 0.11</td>
<td>14.25 ± 1.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.32 ± 0.18</td>
<td>0.77 ± 0.05</td>
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<tr>
<td></td>
<td>Obese</td>
<td>0.73 ± 0.01</td>
<td>23.99 ± 7.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.20 ± 0.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.86 ± 0.11</td>
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<sup>a</sup>Values represent mean fold increase over uninfected control ± SEM (n = 4–6).

<sup>b</sup>p < 0.05 compared with 0 dpi within same dietary group. There were no statistical differences between lean and obese mice at any time point.
Tregs have been shown to regulate all of these parameters in other respiratory infection models (55, 56). Further, obesity has been reported to result in a deficiency of anti-inflammatory Tregs in metabolic tissues such as white adipose (57) and the liver (58). We therefore proceeded to investigate this unique cell type in the secondary infection model as a potential mechanism for increased inflammation in the obese mice. Fig. 7A represents the gating scheme used to distinguish Tregs and demonstrates the Treg distribution in the lung airways and mLNs in lean and obese mice at 5 dpi. Although CD25$^{+}$Foxp3$^{+}$ Tregs were present in the lung airways and mLNs following the secondary infection, the majority of Foxp3$^{+}$ T cells were CD25$^{+}$ (Fig. 7A). BAL Treg number peaked at 5 dpi in obese mice and continually decreased in number, whereas lean mice had consistent numbers of BAL Tregs from 5 to 8 dpi, with a similarly low level of Tregs by 14 dpi (Fig. 7B). Of note, obese mice had more than twice as many BAL Tregs at 5 dpi and nearly half as many mLN Tregs (Fig. 7C) as did lean mice. Consistent with two recent publications investigating Tregs during a primary influenza infection (59, 60), the mLNs in lean and obese mice contained a greater number of Tregs than did the BAL compartment following infection (Fig. 7C). Little is known regarding memory Tregs during influenza infection or the distribution and function of Tregs during a secondary influenza infection. One would expect a greater presence of suppressive Treg cells to result in a dampening of the inflammatory and cytotoxic responses in the lungs of obese mice (61). However, we observed...
populations were lean and obese mice. Fig. 8A demonstrates that isolated Treg nate any possibility of a discrepancy in Teff proliferation between cocultured only with Teff cells isolated from lean mice to elimi-

isolated from lean mice. Tregs from lean and obese mice were 41% less suppressive (Fig. 8C). Therefore, diet-induced obesity mice were found to be significantly less suppressive (Fig. 8B). After normalizing to lean Treg activity, Tregs isolated from obese mice were 41% less suppressive (Fig. 8C). Therefore, diet-induced obesity dramatically impaired Treg suppressive activity. A wide variety of mechanisms could contribute to the impaired Treg function in obese mice. The level of Foxp3 expression in Treg cells is important for suppressive activity, as experimental re-
duction in Foxp3 results in impaired Treg suppressive function (62, 63). Thus, we assayed Foxp3 protein expression levels in Tregs from splenocytes of lean and obese mice, but we did not detect differences in Foxp3 levels (Fig. 8D), suggesting that im-

pairment in Treg function is due to a mechanism unrelated to Foxp3 expression.

Discussion

Globally, > 1.4 billion adults are overweight, and ~ 500 million of these individuals are obese (36). In 2009, obesity was reported to be an independent risk factor for hospitalization and death following pH1N1 influenza infection (32–34). Given the prevalence of obesity and the consistent threat of influenza epidemics and pandemics, understanding how excess adiposity affects the immune response to influenza infection is important in potentially developing therapies to limit morbidity and mortality in this at-risk population.

During the 2009 pandemic, a majority of individuals (non-

elderly) lacked pre-existing neutralizing Ab protection to pH1N1 (10, 12–15). Therefore, a number of investigations have focused on the protectiveness of heterologous immunity to pH1N1 infec-
tion, conferred from previous exposure to circulating influenza strains or vaccines (19, 27, 28). Human studies and mouse models have demonstrated that cross-reactive T cells and cross-reactive nonneutralizing Ab defenses can limit viral load and protect from a lethal heterologous influenza infection (10, 19, 21, 30). In ad-

dition, prior heterologous infection can accelerate pH1N1 Ab generation during a secondary pH1N1 infection, thus providing an additional mechanism by which heterologous immunity can limit pH1N1 infection severity (25, 30). However, obese humans and mice have been shown to have impaired memory T cell and hu-
moral responses following vaccination or infection (38–40, 64). Thus, we developed a mouse model to investigate whether obesity results in enhanced pH1N1 infection severity owing to impair-

ments in heterologous immune defenses.

In this study, obesity did not impair the overall ability of het-

erologous immunity to prevent mortality from pH1N1 infection, as 95% of PR8-primed obese mice survived the lethal secondary heterologous infection, whereas none of the naive, obese mice survived the pH1N1 infection. However, heterologous cellular and humoral responses were significantly altered by obesity, and obese mice displayed greater lung damage during the secondary pH1N1 infection. Obese mice exhibited a significant delay or impairment in Ab generation during both primary and secondary influenza infections. Further, obese mice displayed significantly lower levels of cross-reactive anti-pH1N1 NP Abs following the primary PR8 infection. Subsequent investigation of viral titers confirmed that obese mice had a greater lung viral titer at 5 dpi, perhaps owing to reduced levels of cross-reactive anti-NP Abs. Consistent with greater viral load, obese mice had an elevated number of infiltrating cells into the lung airways, heightened lung disease, and greater BAL total protein and albumin levels. Analysis of cross-reactive anti-NP Abs resulted in greater lung viral burden, which subsequen-
tly induced greater immune cell infiltration, lung dam-
age, and heightened CD8+ T cell responses in obese mice. Finally, we investigated the impact of obesity on Treg distribution during the secondary infection model. We found that obese mice had a greater number of BAL Tregs at 5 dpi, but ex vivo analysis revealed Tregs isolated from obese mice were significantly less functional than were Tregs from lean mice. Therefore, perhaps

FIGURE 7. Obese mice have a greater number of Tregs in the lung airways during a heterologous secondary pH1N1 challenge. (A) Representative BAL (n = 5–6) and mLN (n = 10–12) CD4+CD25+Foxp3+ Treg gating scheme at 5 dpi, including mean percentage (SEM). (B and C) Enumeration of Tregs in BAL fluid (B) and mLNs (C). Each bar represents the mean ± SEM; *p < 0.05 compared with lean mice at 5 dpi.
obese individuals experienced greater pH1N1 infection severity owing to a combination of impaired pH1N1 heterologous Ab defenses and excessive antiviral immune responses in the lungs, which could not be controlled properly because of dysfunctional Tregs.

Despite a lack of neutralizing Ab protection, Ab defenses may still impart protection to a lethal pH1N1 infection in our model. A protective role for cross-reactive nonneutralizing Abs during influenza infection has been well established (29, 30, 50, 51). LaMere et al. (29) demonstrated that intact Ab defenses (i.e., somatic hypermutation and class-switch recombination) were required for proper heterologous immunity in the classical ≥31 prime, PR8 challenge, heterologous infection model. Further, influenza NP vaccination or passive transfer of NP immune serum can limit viral replication in the lungs of PR8-infected mice (29). Therefore, nonneutralizing Abs can impart antiviral activity, although the exact mechanisms by which anti-NP Abs function remain unclear (29). Of interest, NP was detected in the 2008/09 trivalent inactivated vaccine, and some individuals demonstrated increased levels of anti-NP Abs following vaccination (51). Therefore, nonneutralizing NP Ab protection is clinically relevant as well. The nonneutralizing influenza Ab repertoire can also include Abs targeting other internal viral proteins, such as the matrix protein and nonstructural protein 1 (29, 30). However, the protective role of Abs specific to these proteins is less established and likely less protective compared with anti-NP Abs (29, 30).

Another mechanism by which heterologous immunity may protect against pH1N1 is through the acceleration of homologous pH1N1 Abs produced during a secondary pH1N1 challenge. A primary heterologous infection followed by a pH1N1 challenge has been shown to result in significantly enhanced production of pH1N1-neutralizing Abs (25), split pH1N1 Abs, and anti-NP Abs when compared with production in naïve mice infected with pH1N1 (30). It is likely that cross-reactive CD4+ T cells facilitate this accelerated homologous pH1N1 Ab production (25). We did not detect pH1N1 HAI Abs at the early time point of 5 dpi; however, we found that a significantly lower proportion of obese mice exhibited detectable pH1N1 HAI Abs 14 d after the secondary challenge. Therefore, it is unlikely that this aspect of heterologous immunity contributed to the observed outcomes in lean and obese mice. Of particular note, we found that obese mice displayed a delayed production and impaired maintenance of PR8 Abs following the primary, sublethal PR8 infection. Further, obese mice had significantly lower mean PR8 HAI titers during the secondary infection, and a lower proportion of obese mice had detectable PR8 HAI Abs at all of the time points assayed. Although PR8 HAI Abs did not mediate protection in this model per se, the dramatic impact of obesity on influenza Ab responses has clear public health implications. Kim et al. (40) demonstrated that obesity impairs the Ab response to pH1N1 vaccination in mice, but it has not been reported that obesity alters Ab responses during the context of an infection.

Excessive antiviral and inflammatory immune responses in the lung, contributing to severe immunopathology, are known to be one of the primary causes of influenza-related morbidity and mortality (65, 66). Further, fatal pH1N1 infection is associated with enhanced inflammation with high numbers of CD8+ GzB+ cytotoxic T cells and excess local production of IFN-γ in the lung (66). We similarly detected greater inflammation, more lung disease, and a greater number of CD8+ GzB+ cytotoxic T cells in the lungs of obese mice infected with pH1N1. Although only mild symptoms were experienced by a majority of individuals infected, severe immunopathology may partly explain why certain at-risk groups, such as obese humans, experienced a worse outcome to pH1N1 infection.

Obesity is characterized by a state of chronic inflammation, likely owing, in part, to the simultaneous deficiency of Tregs in metabolic tissues (57). Because obesity negatively affects Treg distribution, we hypothesized that obese mice would have fewer Tregs in their lungs, offering a potential explanation for the greater lung infiltration, inflammation, disease, and damage, as well as the heightened memory CD8+ T cell responses detected in the lung airways at 5 dpi. Tregs have been shown to regulate all of these parameters in other respiratory infection models (55, 56, 67). Contrary to our expectations, we found that obese mice had nearly twice as many Tregs in the lung airways as did lean mice. Subsequent investigation of Treg function revealed that Tregs isolated from obese mice were 41% less suppressive than those isolated from lean mice. Therefore, impaired Treg function may have contributed to the excessive antiviral responses observed in the lungs of obese mice at 5 dpi. Although we did not show impaired Treg suppressive function in vivo during the infection, the suppression assay suggests that in vivo function may be impaired as well.

Betts et al. (60) demonstrated that a primary influenza infection results in a robust, Ag-specific Treg response, and influenza infection–induced Tregs were able to suppress Ag-specific CD4+ and CD8+ T cell proliferation and cytokine production. Further, a recent study by Brinks et al. (68) reported that during secondary influenza infections, Ag-specific memory Tregs exhibit accelerated accumulation in the lungs and mLNs, compared with primary infection responses and control memory CD8+ T cell proliferation in a MHC class II–dependent Ag-specific manner. Of interest, depletion of memory Tregs resulted in heightened CD8+ T cell...
responses, pulmonary inflammation, and lung cytokine expression during a secondary influenza infection (68). It is possible that obesity-induced alterations in memory Treg responses could have contributed to the aberrant inflammatory responses observed in obese mice during the secondary pH1N1 infection.

We used a mouse model of diet-induced obesity and heterologous influenza infection in an attempt to mirror clinical and epidemiological evidence. We found lower levels of nonneutralizing influenza NP Abs, heightened cross-reactive CD8+ T cell responses, greater viral titers and lung disease, and impaired Treg function in obese mice. We propose that impaired Ab protection resulted in greater viral burden, which subsequently drove heightened inflammatory responses that were not properly controlled by dysfunctional Tregs. This study and numerous others have demonstrated that obesity negatively affects the immune response to influenza infection (38, 40, 42, 64, 69–71). Yet, the exact cellular and molecular mechanisms by which excess adiposity contributes to altered antiviral defenses remain unclear (37). It is possible that one global defect in all immune cells can explain impaired functionality in the obese, but it is more likely that a complex combination of altered signaling pathways and responses ultimately impairs immunity and results in greater influenza susceptibility (37).

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Disclosures

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References


