Diet-induced obesity in mice causes changes in immune responses and bone loss manifested by bacterial challenge

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Obesity has been suggested to be associated with an increased susceptibility to bacterial infection. However, few studies have examined the effect of obesity on the immune response to bacterial infections. In the present study, we investigated the effect of obesity on innate immune responses to *Porphyromonas gingivalis* infection, an infection strongly associated with periodontitis. Mice with diet-induced obesity (DIO) and lean control C57BL/6 mice were infected orally or systemically with *P. gingivalis*, and periodontal pathology and systemic immune responses were examined postinfection. After oral infection with *P. gingivalis*, mice with DIO had a significantly higher level of alveolar bone loss than the lean controls. Oral microbial sampling disclosed higher levels of *P. gingivalis* in mice with DIO vs. lean mice during and after infection. Furthermore, animals with DIO exposed to oral infection or systemic inoculation of live *P. gingivalis* developed a blunted inflammatory response with reduced expression of TNF-α, IL-6, and serum amyloid A (SAA) at all time points compared with lean mice. Finally, peritoneal macrophages harvested from mice with DIO and exposed to *P. gingivalis* exhibited reduced levels of proinflammatory cytokines compared with lean mice and when exposed to *P. gingivalis* LPS treatment had a significantly reduced recruitment of NF-κB to both TNF-α and IL-10 promoters 30 min after exposure. These data indicate that obesity interferes with the ability of the immune system to appropriately respond to *P. gingivalis* infection and suggest that this immune dysregulation participates in the increased alveolar bone loss after bacterial infection observed in mice with DIO.

cytokine | *P. gingivalis* | inflammatory response | macrophage | chromatin immunoprecipitation

Periodontal disease is a common infectious disease associated with Gram-negative anaerobic bacteria such as *Porphyromonas gingivalis* and characterized by inflammation and destruction of periodontal tissues. Within this privileged site, *P. gingivalis* can replicate and impinge upon components of innate host defense. In addition, although periodontal disease is localized to the tissues surrounding the teeth, epidemiologic evidence has suggested that infection with *P. gingivalis* is also linked to more serious systemic conditions such as cardiovascular disease, diabetes, and obesity, and even complications of pregnancy, including premature rupture of the membranes and subsequent delivery of low-birth-weight babies (1–6).

In the last decade, several epidemiological studies have found an association between obesity and an increased incidence of periodontal disease (7–11). Among people with periodontal disease, obesity is associated with deep periodontal pockets, and body mass index (BMI) is positively correlated with the severity of periodontal attachment loss (8, 11). Individuals who maintained a normal weight, pursued regular exercise, and consumed a diet in conformity with the Dietary Guidelines for Americans and the Food Guide Pyramid recommendations were 40% less likely to have periodontitis (12–15). Moreover, obesity significantly contributed to the severity of periodontal disease in an animal model (16). Using a ligature-induced periodontitis rodent model, Perlstein *et al.* (16) found that alveolar bone resorption was greater in obese compared with nonobese rats. These observations suggest a connection between obesity and periodontitis; however, the underlying mechanisms and the role of the peripheral immune response to chronic infections (e.g., periodontal disease) in obese animals are poorly understood.

Obesity has recently been reported to be associated with immune dysfunction (17, 18). In humans, circulating proinflammatory cytokines have been detected in obese individuals, suggesting a state of dysregulated inflammatory response. In both genetic and diet-induced animal models, obesity has been reported to be associated with immune dysfunction (19, 20). *In vitro*, the secretion of inflammatory cytokines such as IL-4 and IFN-γ was found to be impaired (21).

The causal relationship(s) between obesity and periodontitis and potential underlying biological mechanisms remains to be established, but with 30% of the population in the United States obese and severe generalized periodontal disease affecting >20% of that population, it is important to understand how obesity may impact a person's ability to respond to infection (22).

In the present studies, we tested our hypothesis that diet-induced obesity (DIO) impairs the host immune response to bacterial infection and leads to more severe periodontitis and alveolar bone loss in obese animals. By inoculating *P. gingivalis* locally into the oral cavity or systemically in lean mice and mice with DIO, we found that DIO affects the immune response to *P. gingivalis* challenge and subsequent infection, which in turn increases periodontal morbidity.

Results

Mice DIO. After 16 weeks, normal C57BL/6 mice on high-fat diet (HFD) gain ≈50% more body weight than standard chow diet (SCD) mice (Fig. 1*A*). The mice fed with HFD ingested 12 times more calories than lean mice, even though their daily food intake did not differ appreciably (Fig. 1*B*). However, blood glucose levels were found not to differ significantly between mice with DIO and lean mice (Fig. 1*C*). Therefore, the DIO mice had not yet developed to diabetes (serum glucose level <125 mg/dl).

DIO Leads to Increased Alveolar Bone Loss in a Murine Model of Periodontal Disease. To assess the role of DIO in periodontitis, we subjected lean mice and mice with DIO to experimental periodontal disease and analyzed alveolar bone loss. Experimental peri-

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The authors declare no conflict of interest.

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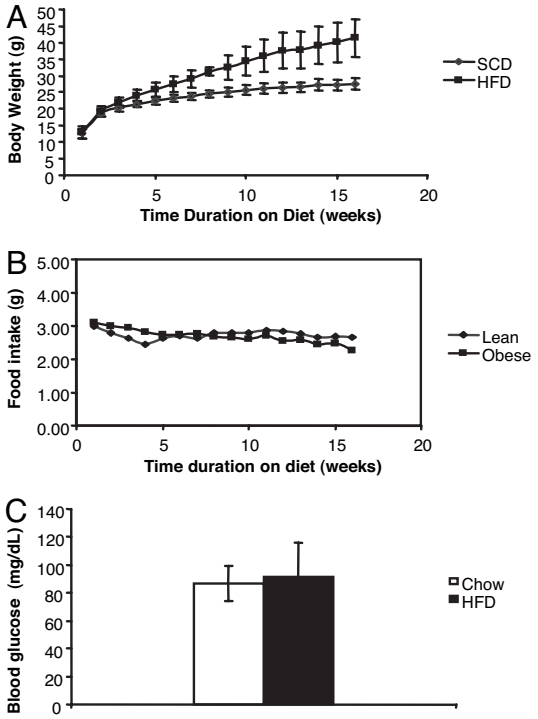

Fig. 1. DIO mouse model used in the study. Four-week-old male Jackson Laboratory C57BL/6 mice were fed either with SCD or HFD for 16 weeks. Body weight was measured weekly (*A*). Mean food intake (*B*) and serum glucose levels (*C*) were measured before experiments.

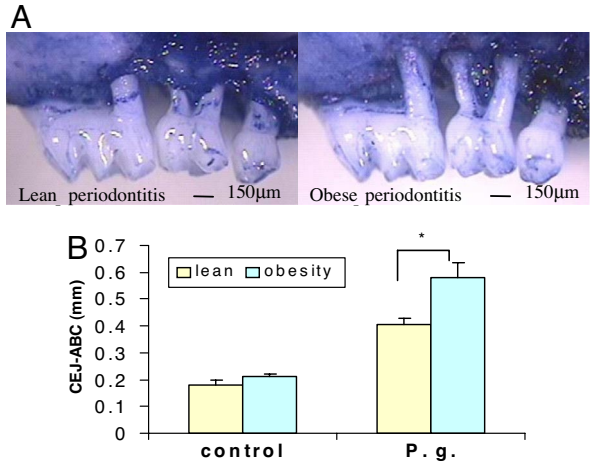

Fig. 2. *P. gingivalis*-induced alveolar bone loss in experimental periodontitis in both lean mice and mice with DIO. Mice from both groups were exposed to *P. gingivalis*-soaked or broth-soaked ligatures. After four challenges with bacteria or broth alone, mice were euthanized, bone tissue was prepared, and bone measurements were performed by morphometric analysis (*A*). Distance from the cemental enamel junction (CEJ) to the alveolar bone crest (ABC) was measured in millimeters, and the data presented are means ± SEM (*B*) ($n = 5$ for each group). *, $P = 0.0007$.

odontitis was induced by wrapping *P. gingivalis*-soaked ligatures around the maxillary second molar. The *P. gingivalis* infected DIO group showed a 40% increase in alveolar bone loss compared with their lean counterparts ($P = 0.0007$), whereas uninfected lean mice and mice with DIO did not exhibit any significant bone loss (Fig. 2).

Mice with DIO Exhibit Higher Titers of *P. gingivalis*. We used *P. gingivalis*-induced experimental periodontitis to evaluate the ability of *P. gingivalis* to infect and survive in mice with DIO. Although no significant differences in total aerobic or anaerobic bacteria were observed between lean mice and mice with DIO (data not shown), higher levels of *P. gingivalis* were observed in plaque samples from mice with DIO compared with lean mice throughout the experiment ($P < 0.05$) (Table 1) with a maximum colony-forming unit (CFU) on day 10. Interestingly, although the rate of *P. gingivalis* log CFU increase (5%) was similar between DIO and lean animals until day 10, the rate of elimination was greater in lean vs. DIO animals (20% vs. 8%, respectively). These results indicate that mice with DIO were unable to clear *P. gingivalis* as efficiently as lean mice and suggest that DIO impairs the mechanisms associated with host removal of *P. gingivalis* from the oral environment (Table 1).

Reduced Serum TNF-α Levels in Mice with DIO During Experimental Periodontitis. To determine the effect of *P. gingivalis* infection on serum cytokine levels in mice with DIO, serum levels of TNF-α were quantified by ELISA during *P. gingivalis*-induced periodontitis. In response to *P. gingivalis* infection, lean mouse TNF-α level increased from day 5 with maximum response on day 7. However, in mice with DIO, TNF-α response was delayed until day 7 and was significantly blunted on days 7, 10, and 12 (Fig. 3) compared with lean mice. These results indicate that mice with DIO exhibit an attenuated systemic immune response to *P. gingivalis*-induced periodontitis, supporting our hypothesis that DIO alters innate immune response to *P. gingivalis*, leading to impaired elimination of *P. gingivalis* (Table 1) and aggravated periodontal bone loss (Fig. 2). Uninfected lean mice and mice with DIO did not exhibit any significant changes in TNF-α level.

DIO Affects Host Immune Response to Bacterial Infection *in Vivo*. We tested the effects of DIO on the systemic immune response to chronic bacterial infection. Mice with DIO and lean mice were challenged intravenously with live *P. gingivalis* or vehicle only by tail vein injection, and proinflammatory cytokines (TNF-α and IL-6) in the sera were measured by ELISA. On day 5 in lean animals, substantial TNF-α and IL-6 responses were observed 1 h after *P. gingivalis* injection, and these responses were quickly reduced toward baseline 2 h later. However, in mice with DIO, a blunted TNF-α and IL-6 response to *P. gingivalis* was observed 1 h after injection, and the response remained stable for 2 h (Fig. 4 *A* and *B*). A similar pattern was observed for the acute phase protein serum amyloid A (SAA), a marker of systemic inflammation, although it was slightly more elevated in unchallenged mice with DIO at day 0 (Fig. 4*C*). Vehicle-injected animals did not exhibit any significant changes in TNF-α, IL-6, and SAA.

DIO Affects Macrophage Response to *P. gingivalis* Infection *in Vitro*: Bio-Plex Cytokine Array. To investigate the innate immune response in peripheral macrophages from DIO vs. lean mice, levels of 23 cytokines were analyzed by Bio-Plex cytokine array using supernatants from macrophages obtained from mice with DIO and lean mice, 15 min and 4 h after *ex vivo* challenge with live *P. gingivalis* or PBS only. Among the cytokines analyzed in this experiment, TNF-α, IL-1α, IL-1β, IL-6, MIP-1α, MIP-1β, RANTES, MCP-1, KC, GM-CSF, G-CSF, and IL-12p40 were significantly reduced in macrophage cultures from mice with DIO compared with lean mice (Fig. 5). A similar observation was made when macrophages were exposed to *P. gingivalis* LPS: Although DIO macrophages exhibited higher baseline levels of TNF-α compared with lean macrophages, they expressed 50% and 60% less TNF-α after 4-h and 24-h LPS exposure, respectively, than lean macrophages (Fig. 6). PBS-exposed lean macrophages and macrophages from DIO did not exhibit any significant changes in cytokine profile (Figs. 5 and 6).

Table 1. Log *P. gingivalis* CFUs from subgingival samples at time points after the first ligature placement

	P. gingivalis cultured from gingival samples, average log CFU ± SD ($n = 6$)							
Mice	Day 0	Day 3	Day 5	Day 7	Day 10	Day 12	Day 14	Day 16
Lean	0	3.09 ± 1.29	4.14 ± 0.06	3.99 ± 0.25	4.68 ± 0.12	2.79 ± 0.61	2.14 ± 0.3	1.22 ± 0.81
Obese	0	3.58 ± 0.14	4.68 ± 0.07	4.53 ± 0.13	5.16 ± 0.06	3.26 ± 0.27	3.06 ± 0.09	2.75 ± 1.15

Statistical significance ($P < 0.05$) was found at each point, except day 0 (not determined).

NF-κB Pathway-Focused cDNA Array. To determine the effect of DIO on gene expression of NF-κB-related signaling molecules, peritoneal macrophages obtained from lean mice and mice with DIO were challenged with *P. gingivalis* LPS for 4 h. When macrophages were challenged with *P. gingivalis* LPS for 4 h, 13 genes were differentially expressed between cells derived from DIO and lean animals. The expression levels of genes known to play a role in inflammation, such as *IL1r1*, *Traf3*, *Rel*, *Tlr4*, and *Nfkb1*, were higher in DIO macrophages than lean macrophages, whereas the expression levels of genes counteracting these factors, such as *raf6*, *Nfkbia*, *Csf3*, *Icam1*, *Ripk1*, *Rela*, *Tnfaip3*, and *Traf5*, were lower in DIO, supporting our hypothesis that the host immune response to *P. gingivalis* is dysfunctional in DIO compared with lean animals (Fig. 7).

Chromatin Immunoprecipitation (ChIP). To further characterize the DIO immune dysregulation, we evaluated the modifications of DIO to chromatin at two representative loci over time: a classic proinflammatory locus (TNF promoter) and an equally classic antiinflammatory locus (IL-10 promoter). The effect of *P. gingivalis* LPS on the recruitment of NF-κB to the TNF-α and IL-10 promoters was assessed in DIO vs. lean mice. Recruitment of NF-κB to both TNF-α and IL-10 promoters in macrophages from lean mice at 30 min was readily detected, whereas this recruitment was substantially reduced in macrophage from mice with DIO (Fig. 8).

Discussion

DIO effects on immune function are poorly understood. Although genetically obese mice harboring a leptin defect (*ob*/*ob* or *db*/*db*) have been shown to exhibit an impaired immune function (19, 23), there are not appropriate models to study innate immunity because the phenotype of the leptin defects includes impaired immune function. We selected the DIO model because most obese individuals suffer from over-nutrition and only a small number of individuals are obese due to mutations in the leptin gene (24). In our model of periodontal disease, the effect of DIO was surprisingly dramatic. Mice with DIO exhibited a 40% increase in bone loss 10 days after the first bacterial inoculation, a time frame consistent with pronounced changes in immune response. Accompanying the increase in bone loss was an altered systemic immune response, including a blunted proinflammatory cytokine expression in mice with DIO compared with lean animals. This blunted response was observed in all serum samples whether after direct systemic inoculation or after oral inoculation of *P. gingivalis*. Congruent with our results, Smith *et al.* (17) showed recently that mice with DIO harbor an increased mortality and altered immune responses when infected with influenza virus. To our knowledge, this is the first report of DIO interfering with normal host responses to bacterial infection.

This blunted inflammatory response was observed for TNF-α, IL-6, and SAA. However, although no constitutively elevated TNF-α and IL-6 levels were observed in unchallenged animals with DIO, SAA, an acute phase protein, was slightly elevated in unchallenged mice with DIO, substantiating recent reports suggesting that obese animals exhibit an elevated inflammatory response at baseline even unchallenged (25).

In addition to the blunting of the inflammatory response, bacterial counts for *P. gingivalis* were elevated in mice with DIO

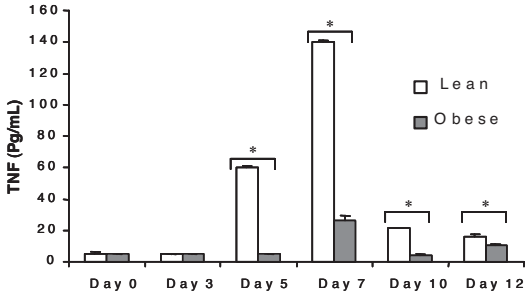

Fig. 3. TNF-α levels in sera from mice with experimental periodontitis induced by *P. gingivalis*. Groups of DIO and lean mice were infected with *P. gingivalis* in the form of ligature insertions at the second molar on day 0, and ligatures were replaced on days 3, 5, and 7. Blood samples were collected before ligature insertion at days 0, 3, 5, 7, 10, and 12. TNF-α levels in the sera were determined by ELISA (BioSource). *, $P < 0.05$.

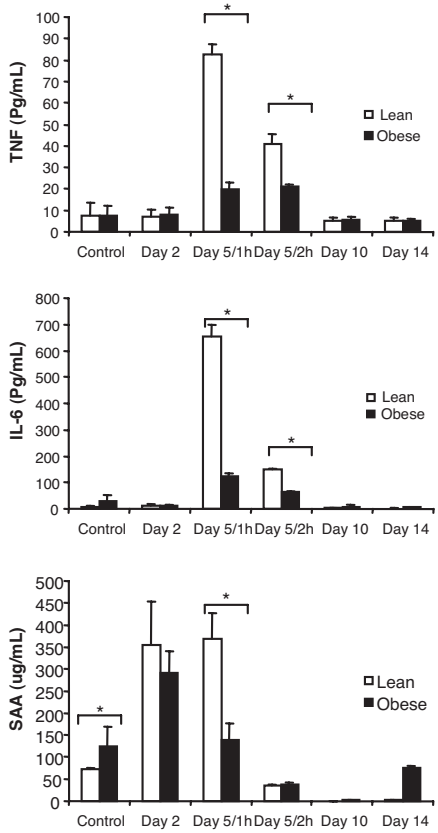

Fig. 4. Serum TNF-α (*A*), IL-6 (*B*), and SAA (*C*) levels in response to infection with *P. gingivalis* strain A7436 in lean and mice with DIO by the intravenous route. Mice were infected with *P. gingivalis* (2.0×10^9 in 50 μl) or vehicle by tail vein injection on days 1, 3, and 5. TNF-α, IL-6, and SAA concentrations in serum were measured by ELISA (means ± SEM, $n = 8$). *, $P < 0.05$.

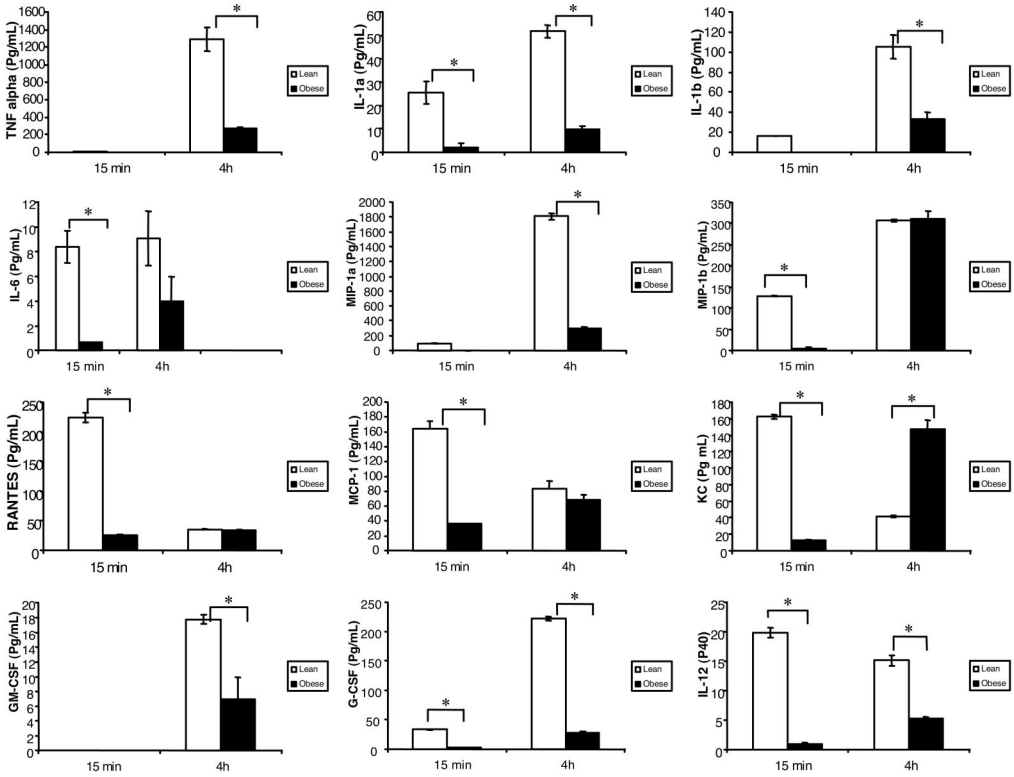

Fig. 5. Global cytokine profile in response to *P. gingivalis* stimulation *in vitro*. Macrophages from lean mice and mice with DIO were cultured and exposed to live *P. gingivalis* for 15 min and 4 h (MOI = 25:1). Cells treated with PBS were used as controls. Culture supernatants were analyzed by a Bio-Plex cytokine array. Cytokines induced after 15-min and 4-h treatment and their average concentrations (pg/ml) (means ± SD; $n = 3$) are shown. *, $P < 0.01$ between lean and DIO. Cytokines that did not change significantly between lean and obese were the following: eotaxin, IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-12p70, IL-13, and IL-17.

compared with lean animals whether during the oral inoculation period or after. These elevated bacterial counts in mice with DIO could stem from a host's inability to mount an adequate inflammatory response as evidenced by the blunted response. This inability to mount an adequate inflammatory response would not allow proper elimination of the bacteria, which would in turn linger in the oral sites longer and cause more direct damage including bone loss. A similar observation was made in Leptin-deficient mice harboring an impaired host defense to bacterial pneumonia (26). Moreover, the ability of mature macrophage to elicit antimicrobial and cytotoxic responses may be inhibited in mice with DIO compared with lean animals (27). Based on these data, the use of antiinflammatory agents in DIO individuals with severe infection may not be appropriate; rather, strong antimicrobial should be advocated given the bacterial burden.

In vitro, peritoneal macrophages from animals with DIO exposed to *P. gingivalis* demonstrated a similar blunting of the prominent proinflammatory cytokines TNF-α, IL-1β, IL-6, IL-12, RANTES and MCP-1, compared with lean animals. *P. gingivalis* LPS appears to be a potential virulent factor affecting this blunting because peritoneal macrophages from DIO animals exposed to *P. gingivalis* LPS also expressed lower levels of TNF-α. Given that *P. gingivalis* LPS is a specific TLR2 ligand (28, 29, 35), the reduced TNF-α expression may stem from dysregulated TLR2 signaling possibly involving NF-κB, as evidenced by the NF-κB pathway-focused cDNA arrays. We tested the recruitment of NF-κB onto the promoters of a pro- and antiinflammatory cytokine: TNF-α and IL-10. For both mediators, known to be activated via TLR2, the recruitment was drastically reduced at 30 min, demonstrating that not only might the proinflammation process be dysregulated but also the antiinflammation process. The lack of inflammatory gene

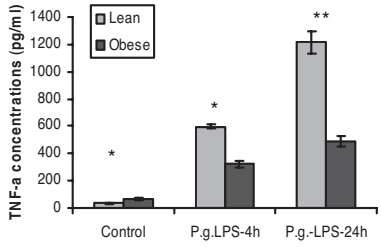

Fig. 6. Effects of DIO on macrophage response to *P. gingivalis* LPS. Mouse macrophages isolated from lean mice and mice with DIO were challenged with PBS or *P. gingivalis* LPS for 4 h and 24 h. TNF-α concentrations in cell culture supernatants were measured by ELISA ($n = 3$). *, $P < 0.05$; **, $P < 0.001$.

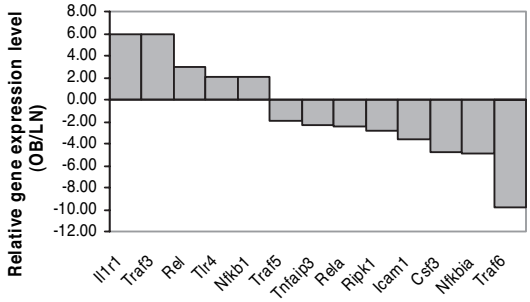

Fig. 7. Differential gene expression between macrophages from mice with DIO and lean mice after *P. gingivalis* LPS challenge. Data show the relative gene expression level in DIO over that from lean mice. Negative values signify gene expression lower in DIO mouse macrophages compared with lean counterparts.

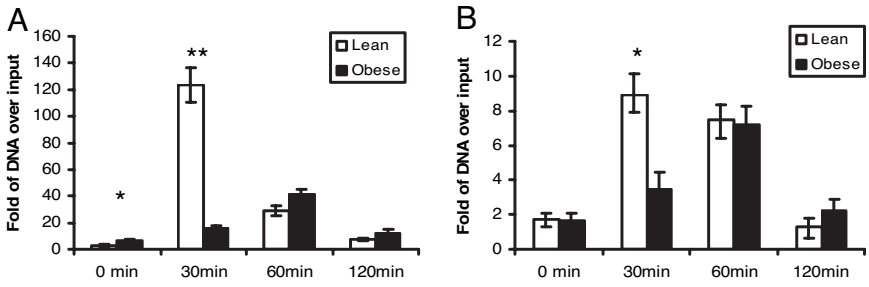

Fig. 8. Early and transient recruitment of NF-κB to the TNF-α and IL-10 promoters. Peritoneal macrophages from lean mice and mice with DIO were treated with *P. gingivalis* LPS (10 μg/ml) for the indicated times, and ChIP assays were performed with an anti-p65 affinity-purified rabbit polyclonal Ab. p65-precipitated DNA was analyzed by quantitative real-time PCR with promoter-specific primers amplifying the TNF-α and IL-10 promoters. The results are expressed as means ± SD; $n = 4$ for each time point. *, $P < 0.05$; **, $P < 0.001$.

expression is balanced by a concomitant reduction in IL-10 expression, indicating that systemic circulation and the periodontium of mice with DIO were in a dysregulated inflammatory state. Another possible explanation for reduced cytokine expression in mice with DIO is a reduction in number and/or maturation of macrophages in the circulation as well as the periodontal area during infection. Indeed, obese humans have similar numbers of circulating monocytes, but the number of monocytes that matured into macrophages was found to be almost three times less in these individuals (30). In addition, the ability of mature macrophages to elicit an antimicrobial and cytotoxic response may be inhibited (27). Because macrophages are a major contributor to proinflammatory cytokine production, fewer macrophages in circulation and in the periodontium, as well as a decrease in their functional capacity, could explain the reduction in cytokine levels. Finally infiltration of monocytes may also be reduced in the mice with DIO because they expressed significantly lower levels of MCP-1.

The importance of the current findings is underscored by the facts that millions of people worldwide are affected by *P. gingivalis* infection every year and the universal prevalence of obesity has reached epidemic proportions. In this study, we found that DIO led to dysregulated innate immune responses to *P. gingivalis* infection and increased morbidity. Furthermore, these data suggest that, in addition to *P. gingivalis* infection, DIO may increase susceptibility to other bacterial infections by way of immune system dysregulation.

We propose that in normal mice, a homeostatic cytokine network maintains a regulated response to bacterial challenge through a cycle of transient inflammation, followed by down-regulation with antiinflammatory cytokines. As obesity develops, a dysregulation in this homeostatic network that normally counters inflammation is observed. DIO becomes associated with a form of immune paralysis including an altered pro- and antiinflammatory network in the periphery, an altered gene expression profile in peripheral monocyte/macrophage, an altered capacity for signaling through TLRs and other microbially induced pathways, and an altered chromatin status definable at specific cytokine loci to reflect the cellular context of the inflammatory process.

Methods

Animals and Diets. C57BL/6J mice were obtained from Charles River Laboratories. All mice were housed at the Boston University Medical Center Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. All animal protocols were approved by the Institutional Animal Use and Care Committee. Age, strain, and sex-matched 4-week-old mice were randomly assigned to either a HFD (D12492: 60% kcal fat; Research Diets) or a SCD (2018: 5% kcal fat; Harlan Teklad) for 16 weeks. Body weight of mice was measured weekly. Mice were housed four per cage with free access to food and water, with the exception of an 8-h food deprivation period before blood draws for glucose.

Blood Glucose. Blood glucose concentrations were measured with a Freestyle blood glucose monitor (Abbott Laboratories). Animals with serum glucose levels >125 mg/dl were excluded.

Bacterial Infection. Two models of infection were used.

Systemic infection. Mice with DIO and lean mice were challenged intravenously with live *P. gingivalis* or vehicle (bacteriostatic 0.9% sodium chloride; Hospira) by tail vein injection as reported in ref. 31. *P. gingivalis* (5×10^8 CFUs per mouse each time) or vehicle was injected on days 1, 3, and 5. Venous blood was collected on days 0, 2, 5, 10, and 14. On day 5, blood was collected 1 and 2 h after *P. gingivalis* inoculation, to capture the early host immune activation response and also the return to baseline, both important components of the host response ($n = 8$ for each time point).

Oral infection. Experimental periodontitis was induced in lean and DIO animals by tying a 5-0 silk ligature around both the maxillary right and left second molars, placing the ligatures in the gingival sulcus for 10 days. Ligatures were presoaked in broth containing *P. gingivalis* strain A7436 (10^8/ml), which was cultured as we describe in ref. 32. Ligatures were changed every other day on days 3, 5, and 7 to maintain a sufficient microbial burden. Mice were euthanized on day 10 ($n = 6$). A control group of lean mice and mice with DIO was placed with ligatures presoaked in broth without *P. gingivalis* and processed 10 days later in the same way as the infected group.

Measurement of Bone Levels. Bone tissue was prepared according to Baker *et al.* (33), and bone loss around the roots of mouse teeth (alveolar bone) was measured by morphometric analysis on six tooth aspects: mesio-buccal (MB), mid-buccal (MidB), disto-buccal (DB), disto-palatal (DP), mid-palatal (MidP), and mesio-palatal (MP), as we describe in refs. 32 and 34.

Quantitation of Bacterial Titers. DIO and lean animals ($n = 10$) received ligature placement according to the schedule above. Subgingival plaque samples of left and right maxillary second molars were collected in each group by using sterile paper points before ligature insertion on days 0, 3, 5, and 7 and then after completion of the ligature phase on days 10, 12, 14, and 16. The plaque samples were plated for aerobe and anaerobe plaque analysis as we describe in ref. 32. The total CFUs were determined, and *P. gingivalis* from anaerobic bacterial cultures was identified from colony morphology and biochemical properties by using the API (Anaerobic Pathogen Identification) system (bioMérieux).

Quantitation of Circulating Cytokine Levels. Locally and systemically challenged animals were assayed for circulating TNF, IL-6, and SAA by ELISA. For the oral model, blood samples were collected before ligature placement on days 0, 3, 5, and 7 as described above, and then ligatures were removed on day 10. Blood was also collected on days 10 and 12. For the systemic model, serum samples were collected on days 0, 2, and 5 after 1- and 2-h *P. gingivalis* inoculation, as well as on days 10 and 14.

Macrophage Isolation and Culture. Mouse peritoneal macrophages were isolated by peritoneal lavage, as described in ref. 35. Isolated macrophages were plated into 100-mm cell culture dishes or six-well plates at a concentration of 1×10^6 cells per ml in RPMI medium 1640 supplemented with 10% FBS and standard penicillin/streptomycin. After a 2-h incubation at 37°C in an atmosphere containing 5% CO_2, nonadherent cells were washed out with warm PBS. Adherent macrophages were cultured overnight before experiments. Media were changed 1 h before experiments. Adherent macrophages were infected with live *P. gingivalis* with multiplicities of infection (MOI) of 25:1. Live *P. gingivalis* A7436 frozen stocks were thawed and cultured for 24 h, and then the cultures were collected and diluted in medium to a concentration of 5×10^8 bacteria per 50 μl, to give MOI as indicated, and added to cultures of macrophages. Dilutions were also plated on brain-heart infusion agar plates for anaerobic culture, and colonies were counted to confirm the accuracy of dilution and viability of bacteria. In additional cultures, purified LPS from *P. gingivalis* was added to cell culture medium at a concentration of 10 μg/ml. Cells were incubated at 37°C in an atmosphere containing 5% CO_2 for the indicated times.

Cytokine Profile Analysis. Peritoneal macrophages from mice with DIO and lean mice exposed to live *P. gingivalis* or to PBS were analyzed for levels of 20 cytokines by Bio-Plex cytokine array. At the end of the 24-h stimulation, supernatants from *P. gingivalis*-treated macrophage cultures, as well as PBS-treated macrophage cultures, were collected and analyzed by using a Bio-Plex cytokine reagent kit with Bio-Plex mouse cytokine 20-Plex Panel in the Bio-Plex 200 system (Bio-Rad) as directed by the manufacturer.

NF-κB Pathway-Focused cDNA Array. Peritoneal macrophages obtained from lean mice and mice with DIO were challenged with *P. gingivalis* LPS for 4 h. Total RNA was extracted by using an RNeasy Mini kit (QIAGEN) and hybridized to NF-κB pathway-focused cDNA arrays (GEArray Q Series gene expression array; SuperArray) as directed by the manufacturer. A set of 96 genes associated with NF-κB signaling pathways was analyzed.

Chromatin Immunoprecipitation (ChIP). Peritoneal macrophages (10×10^6 cells per sample) were stimulated, washed with PBS, and fixed with 1% formaldehyde for 10 min at room temperature. Formaldehyde fixation was stopped with the addition of 1.25 M glycine. Fixed cells were sonicated to obtain fragments ranging from 200 to 700 bp in size. Sonicates were diluted five times and incubated with antibody and rotation overnight. Protein A/G beads (Upstate Biotechnology) were added for 3 h, and collected beads were washed extensively. Protein–DNA complexes were eluted from the beads and treated with 200 mM NaCl to reverse cross-links and proteinase K to digest proteins. Recovered DNA was purified by using the GFX PCR DNA and Gel Band Purification kit (Amersham). Immunoprecipitated DNA and input DNA were amplified with gene-specific and GAPDH primers by qPCR, using input DNA to generate a standard curve. ChIP data are represented as percent input.

Statistical Analysis. Statistical analyses were performed by using JMP statistical software (SAS Institute). Normally distributed data were analyzed by two-way ANOVA with diet and infection as main effects. Student's *t* test was used for post hoc comparison between the dietary groups, and Tukey's test was used for post hoc comparisons among the days of infection. Nonparametric data were analyzed by using the Kruskal–Wallis test. Differences were considered significant at $P < 0.05$.

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