MAJOR ARTICLE

Acute *Chlamydia pneumoniae* Reinfection Accelerates the Development of Insulin Resistance and Diabetes in Obese C57BL/6 Mice

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Background. Epidemiological and pathological evidence links highly prevalent pathogens to chronic inflammatory diseases, such as type 2 diabetes. Animal models contribute critically to the mechanistic understanding of infectious enhancement of inflammatory diseases, which share insulin resistance as the central pathophysiological defect.

Methods. With use of a mouse model, we examined insulin resistance progression and the influence of infection (*Chlamydia pneumoniae*–infected vs. uninfected control mice), genetic background (C57BL/6 vs. A/J mice), dietary fat concentration (27% vs. 5%), and time (2, 5, 9, or 15 weeks after inoculation).

Results. In obese C57BL/6 mice, *C. pneumoniae* infection induced significantly increased insulin resistance that persisted long after bacterial clearance. Circulating tumor necrosis factor (TNF)–α produced in response to acute *C. pneumoniae* lung colonization exacerbated insulin resistance but not TNF-α released in situ during secondary chlamydial infection. Azithromycin or anti–TNF-α antibody prevented infection-exacerbated insulin resistance but significantly enhanced chlamydial dissemination to the heart. Azithromycin-treated mice did not eliminate *C. pneumoniae* from lungs by 3 weeks after inoculation but had significantly lower loads (42 genomes per 100 mg) than did control mice (219 genomes per 100 mg) or anti–TNF-α antibody–treated mice (3090 genomes per 100 mg).

Conclusions. Murine *C. pneumoniae* infection enhanced insulin resistance development in a genetically and nutritionally restricted manner via circulating mediators. The relevance for the current human diabetes epidemic remains to be determined, but this finding is potentially important because of the high prevalence of human *C. pneumoniae* infection worldwide.

Obesity-associated chronic diseases, such as type 2 diabetes and atherosclerosis, are driven by inflammatory mediators, such as tumor necrosis factor (TNF)–α [1–4], which are also expressed during infection [5, 6]. Epidemiological and pathological evidence has long linked highly prevalent chronic pathogens (i.e., *Chlamydia pneumoniae, Helicobacter pylori, Porphyromonas gingivalis,* hepatitis C virus, human immunodeficiency virus, influenza virus, cytomegalovirus, and herpes simplex virus type 1) to metabolic syndrome, insulin resistance, type 2 diabetes, and coronary artery disease [7–16]. *C. pneumoniae* infection occurs with high frequency in virtually all humans during their lifetime [17, 18], and numerous studies have demonstrated strong links between *C. pneumoniae* infection and metabolic syndrome, insulin resistance, and coronary artery disease [19–25]. However, the cause and effect relationship has remained inconclusive, and the link was not confirmed in some studies or disappeared after controlling for body weight [23, 26]. Moreover, antibiotic prevention treatment failed to reduce the prevalence of secondary coronary events in large clinical trials, including a 4012-patient trial that tested a 1-year course of weekly azithromycin administration [27–30].

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Table 1. Composition of low-fat and high-fat rodent diets.

Component	Diet, g/kg	
	5% Fat[a]	27% Fat[b]
Casein	275.9	275.9
L-Cystine	4.1	4.1
L-Arginine HCl	9.4	9.4
Maltodextrin	132.0	132.0
Sucrose	476.8	242.9
Soybean oil	20.0	20.0
Coconut oil, hydrogenated	30.0	250.0
Mineral mix, AIN-93G	35.0	46.0
$CaCO_3$	2.3	0.4
Vitamin mix	12.0	15.8
Choline bitartrate	2.54	3.0
Tert-butylhydroquinone	0.01	0.05
Orange dye FD&C	0.1	0.0
Blue dye FD&C	0.0	0.1

[a] Harlan Teklad # TD.02514;
[b] Harlan Teklad # TD.02516.

To investigate a potential mechanistic involvement of a bacterial pathogen in insulin resistance and type 2 diabetes, we examined insulin resistance progression and the influence of infection status (*C. pneumoniae*–infected vs. uninfected control mice), genetic background (obesity-prone C57BL/6 [B6] vs. obesity-resistant A/J mice), dietary fat concentration (27% vs. 5%), and time (2, 5, 9, or 15 weeks after inoculation). Determination of both whole blood glucose and plasma insulin concentrations after bolus administration of glucose (intraperitoneal glucose tolerance test) provides a quantitative measure that allows continuous and accurate assessment of insulin resistance [31, 32]. The results unequivocally confirm that *C. pneumoniae* infection enhances insulin resistance in a genetically and nutritionally restricted fashion in obese B6 mice. The data further indicate that insulin resistance exacerbation is induced by systemic inflammatory mediators emanating from the acute lung challenge infection, rather than from low-level persistent chlamydial organisms dispersed to secondary organs, such as adipose tissue or heart.

METHODS

Mouse strains, diets, and* C. pneumoniae *lung infection. All mouse procedures were approved by the Auburn University Institutional Animal Care and Use Committee. Littermate inbred male A/J or B6 mice (Harlan Sprague Dawley) were weaned at 4 weeks of age onto low-fat (5%) or high-fat (27%), 24% protein, high-sucrose diets (Harlan Teklad), modeled after the diet described by Surwit et al. [33] (table 1). *C. pneumoniae* strain CDC/CWL-029 (ATCC VR-1310) was grown, purified, and quantified as described elsewhere [34]. Mice received an intranasal priming inoculation of 2×10^6 *C. pneumoniae* elementary bodies or mock inoculation with sucrose-phosphate-glutamate buffer in week 6 [5, 34], and reinoculation of 5×10^6 *C. pneumoniae* elementary bodies or sucrose-phosphate-glutamate buffer in week 11 (figure 1*A*).

Intraperitoneal glucose tolerance test. After overnight fasting for 16 h, mice received an intraperitoneal injection of 2.0 mg of glucose per g of body weight. Blood samples were obtained by saphenous vein puncture immediately prior to, and 30, 60, 90, and 120 min after glucose administration. Fresh whole blood samples were used for immediate determination of glucose concentration with OneTouch Ultra Test Strips (LifeScan), and plasma insulin concentration was measured by enzyme-linked immunosorbent assay (ELISA) (Crystal Chem).

TNF-α and nonesterified fatty acids assays. Plasma TNF-α concentration was measured by ELISA (BD Biosciences) with SuperSignal ELISA Femto Maximum Sensitivity Substrate

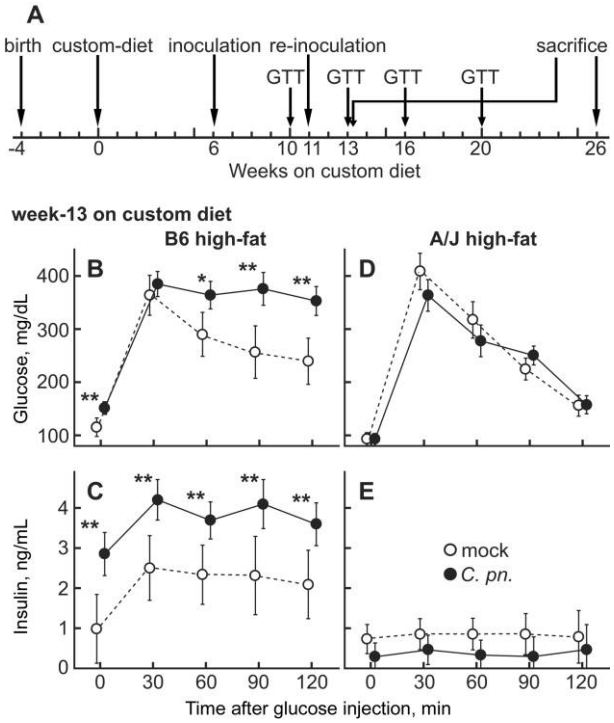

Figure 1. *Chlamydia pneumoniae* infection increases glucose and insulin levels in B6 mice receiving a high-fat diet. Four-week-old mice were weaned onto high-fat or low-fat diets and received a priming inoculation and a reinoculation in weeks 6 and 11, respectively. Glucose tolerance tests (GTTs) were performed in weeks 10, 13, 16, and 20, and the mice were sacrificed in weeks 13 or 26 *(A)*. The week-13 intraperitoneal GTT results demonstrated that *C. pneumoniae*–infected obese B6 mice (*C. pn.*) had significantly higher whole blood glucose levels at 0, 60, 90, and 120 min after glucose injection *(B)* and significantly higher plasma insulin levels *(C)* than did mock-infected mice (mock). Glucose and insulin levels did not differ significantly between mock- and *C. pneumoniae*–challenged A/J mice receiving a high-fat diet *(D* and *E)*. There were 15 mice in each group. The error bars represent 95% confidence intervals. $^*P < .01$; $^{**}P < .001$.

(Pierce). Plasma nonesterified fatty acid concentration was quantified using a colorimetric method (optical density was measured at 570 nm) that measured oxidized fatty acid CoA (BioVision Research Products).

Real-time fluorescence resonance energy transfer (FRET) polymerase chain reaction (PCR) assays. Total nucleic acids and messenger RNA (mRNA) extracted from homogenized mouse tissues were used for quantification of *C. pneumoniae* genomes [34] and transcript concentrations. *C. pneumoniae* genomes were determined using FRET *Chlamydia* 23S ribosomal DNA (rDNA) PCR assays; assays were performed in triplicate to lower the detection limit to 67 genomes per 100 mg of tissue. Murine TNF-α, F4/80, and macrophage chemoattractant protein (MCP)–1 transcripts were quantified relative to the porphobilinogen deaminase reference transcript with use of duplex FRET reverse transcription PCR assays [34].

Anti–TNF-α and azithromycin administration. Immediately prior to and 1 and 2 weeks after mock or *C. pneumoniae* rechallenge in week 11, obese B6 mice received chimeric rat-mouse anti–TNF-α monoclonal antibody cV1q (30 mg per kg of body weight; Centocor) in 200 μL of phosphate-buffered saline by intraperitoneal injection. Alternatively, they received isotype-matched irrelevant murine monoclonal antibody cVam (Centocor) or a subcutaneous administration of azithromycin (120 mg per kg of body weight; Pfizer) combined with an intraperitoneal injection of an irrelevant control antibody.

Statistical analysis. Data were analyzed using factorial and repeated-measures analysis of variance and linear regression. Normal distribution and homogeneity of variances were confirmed by Shapiro-Wilk's *W* and Levene's tests. Comparisons of means under the assumption of no a priori hypothesis were performed using the 2-tailed Tukey honest significant differences test. Data for nonnormally distributed *C. pneumoniae* rDNA copies were analyzed by χ^2 test in which only the presence or absence of *C. pneumoniae* was scored.

RESULTS

C. pneumoniae *infection induces increased insulin resistance in obese B6 mice but not in lean B6 or A/J mice.* We first analyzed the effect of *C. pneumoniae* infection on the development of insulin resistance in the background of genetically obesity-prone (B6) or obesity-resistant (A/J) inbred mice. B6 mice weaned onto a high-fat and high-sucrose diet rapidly become obese, develop insulin resistance and, eventually, type 2 diabetes, whereas A/J mice that are fed the same diet demonstrate less increase in body mass and do not develop type 2 diabetes [33, 35, 36]. Neither mouse strain becomes obese or diabetic when fed a low-fat, high-sucrose diet. In this study, B6 mice weaned onto the high-fat diet had substantially higher body mass than did A/J mice or B6 mice that were fed a low-fat diet. At week 20, the mean body mass of B6 mice receiving a high-fat diet was 44.7 g, which was significantly higher ($P < .001$) than the body mass of B6 mice receiving a low-fat diet (36.0 g) or of A/J mice receiving a high-fat diet (33.3 g) or a low-fat diet (30.3 g).

Five weeks after the mock or priming (2×10^6 *C. pneumoniae* elementary bodies) inoculation, mice received another intranasal mock inoculation or a rechallenge with 5×10^6 *C. pneumoniae* elementary bodies in week 11, to elicit an enhanced inflammatory response [5, 34] (figure 1*A*). *C. pneumoniae*–infected mice did not show signs of disease or a difference in body mass, compared with mock-inoculated controls. Results of intraperitoneal glucose tolerance tests performed 2 weeks after rechallenge clearly indicated reduced glucose tolerance in infected obese B6 mice, which had significantly higher blood glucose levels than did control mice before and 60, 90, and 120 min after glucose administration (figure 1*B*). Even more pronounced was an elevated plasma insulin concentration at all time points (figure 1*C*). In contrast, the glucose and insulin levels of infected A/J mice that were fed a high-fat diet did not differ from that of the A/J control mice (figure 1*D* and 1*E*). These data unambiguously indicate that repeated *C. pneumoniae* infection induced an increase in glucose and insulin levels in obese B6 mice receiving a high-fat diet but not in A/J mice receiving high-fat diet.

Next, we analyzed the influence of dietary fat, genetic susceptibility, and time after *C. pneumoniae* challenge on insulin resistance severity (figures 2 and 3). B6 mice weaned onto a high-fat diet but not B6 mice receiving low-fat diet or A/J mice receiving either diet demonstrated substantially increasing insulin resistance during the observation period of week 10–20 (figure 2*A*–2*D*). After mice received a priming infection in week 6, insulin resistance in week 10 did not differ between *C. pneumoniae*–infected and control mice of both mouse strains receiving a low- or high-fat diet (figure 2*A*–2*D*). *C. pneumoniae* rechallenge in week 11 significantly enhanced insulin resistance in the background of preexisting insulin resistance in obese B6 mice in weeks 13, 16, and 20 (figure 2*A*). The increase in insulin resistance was also significant in lean B6 mice in weeks 16 and 20, but the overall insulin resistance levels in lean B6 mice remained low (figure 2*B*). In contrast, *C. pneumoniae* challenge did not enhance the development of insulin resistance in obesity-resistant A/J mice receiving a high-fat diet (figure 2*C*) or low-fat diet (figure 2*D*). Therefore, challenge with *C. pneumoniae* exacerbates insulin resistance in a genetically and nutritionally restricted manner in B6 mice receiving a high-fat diet but not in B6 mice receiving a low-fat diet or in A/J mice receiving a low-fat or high-fat diet.

C. pneumoniae*–aggravated insulin resistance persists in obese B6 mice after clearance of the bacteria.* The *C. pneumoniae*–enhanced insulin resistance persisted until the end of the observation period in week 26, when challenged obese

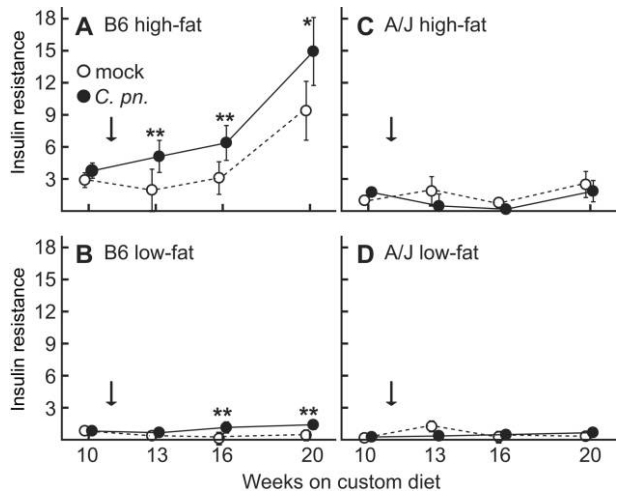

Figure 2. *Chlamydia pneumoniae* infection induces long-term increased insulin resistance in obese B6 mice. Insulin resistance was determined as the product of the total area under the curve (AUC) for blood glucose concentration (mg/dL) and the AUC for plasma insulin concentration (ng/mL) (AUC glucose × AUC insulin). Insulin resistance in week 10 did not differ significantly between *C. pneumoniae*–infected and control mice of both mouse strains receiving a low-fat or a high-fat diet (*A–D*). *A,* Infected B6 mice receiving a high-fat diet showed significantly higher insulin resistance than did mock mice in weeks 13, 16, and 20. *B,* Infected B6 mice receiving a low-fat diet showed significantly increased, but still very low, insulin resistance in weeks 16 and 20. *C* and *D,* Insulin resistance did not differ significantly between *C. pneumoniae*–challenged and mock-challenged A/J mice receiving a high-fat or a low-fat diet. There were 15 mice in each group. The error bars represent 95% confidence intervals. $^{*}P < .05$; $^{**}P < .01$.

B6 mice approached manifest type 2 diabetes (fasted glucose level, 192 mg/dL in obese B6 mice vs. 111 mg/dL in control mice; $P < .001$) (figure 3*A*). Obese B6 mice demonstrated significantly elevated transcript levels of TNF-α (3.44-fold difference; $P = .001$), macrophage marker F4/80 (1.51-fold difference; $P = .05$), and MCP-1 (2.22-fold difference; $P = .05$) in adipose tissue (figure 3*B*). Surprisingly, highly sensitive, triplicate *Chlamydia* 23S rDNA PCR assays did not detect the bacteria in lung, spleen, heart, liver, pancreas, or adipose tissue of any challenged mice in week 26. Similarly, in week 16 or 20, *C. pneumoniae* DNA was not detected in lungs, spleens, or adipose tissue of challenged obese B6 mice that had increased insulin resistance (figure 2*A*). Thus, *C. pneumoniae* infection of obese B6 mice induced increased inflammation of adipose tissue and persistent insulin resistance aggravation that did not disappear after bacterial clearance.

Elevated plasma TNF-α and nonesterified fatty acid levels are associated with increased insulin resistance in* C. pneumoniae*–challenged obese B6 mice. The consensus regarding the mechanism linking obesity to insulin resistance is that adipose tissue is the major source of circulating nonesterified fatty acids, TNF-α, and other proinflammatory soluble factors [1–4, 37]. These factors modify cellular responses to insulin and further enhance their own production, particularly in adipose tissue, in a positive feedback loop [1–3]. Consistent with this concept, the *C. pneumoniae*–induced increased insulin resistance in obese B6 mice (figures 1*B*, 1*C*, and 2*A*) correlated with highly elevated plasma concentrations of TNF-α (figure 4*A*) and nonesterified fatty acids (figure 4*B*) in week 13, 2 weeks after inoculation. These data suggest that *C. pneumoniae* infection triggers the production of TNF-α and nonesterified fatty acids that induce an increase in insulin resistance.

Increased TNF-α transcripts in the lung correlate with high* C. pneumoniae *burden. To examine the origin of the elevated plasma TNF-α level in this mouse model, we analyzed the distribution and levels of TNF-α transcripts and *C. pneumoniae* in organs in week 13. Lungs of infected mice showed a significant 2-fold increase in the number of TNF-α transcripts, compared with lungs of mock-infected mice (figure 4*C*), and the elevated TNF-α transcript levels correlated with high numbers of *C. pneumoniae* genomes in lungs (figure 4*D*). Conversely, the levels of TNF-α transcripts in spleen, heart, pancreas, liver, and adipose tissue of infected mice were not different from that in control mice (figure 4*C*), and *C. pneumoniae* was not detectable or was detectable at very low levels in

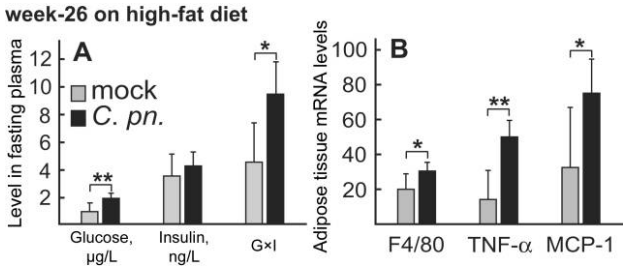

Figure 3. Obese B6 mice exhibit increased glucose levels in plasma and augmented inflammation of adipose tissue after clearance of *Chlamydia pneumoniae.* In week 26, 15 weeks after rechallenge, *C. pneumoniae* was not detected in any of the examined organs (lung, heart, spleen, liver, pancreas, and adipose tissue) of challenged animals, determined by *Chlamydia* 23S ribosomal DNA polymerase chain reactions. An index for insulin resistance (G×I) is calculated as the product of fasting whole blood glucose levels (μg/L) and plasma insulin (ng/mL) concentrations [20] measured in terminal plasma of obese B6 mice after fasting. *A, C. pneumoniae*–challenged obese B6 mice (*C. pn.*) showed significantly increased plasma glucose levels and higher insulin resistance but not plasma insulin levels, compared with mock-infected mice (mock). *B,* Number of F4/80 messenger RNA (mRNA) transcripts per 10 porphobilinogen deaminase (PBGD) transcripts and numbers of tumor necrosis factor (TNF)–α and macrophage chemoattractant protein (MCP)-1 transcripts per 1000 PBGD transcripts were significantly higher in adipose tissue of *C. pneumoniae*–infected mice, compared with mock-infected mice. There were 15 mice in each group. The error bars represent 95% confidence intervals. $^{*}P < .05$; $^{**}P < .01$.

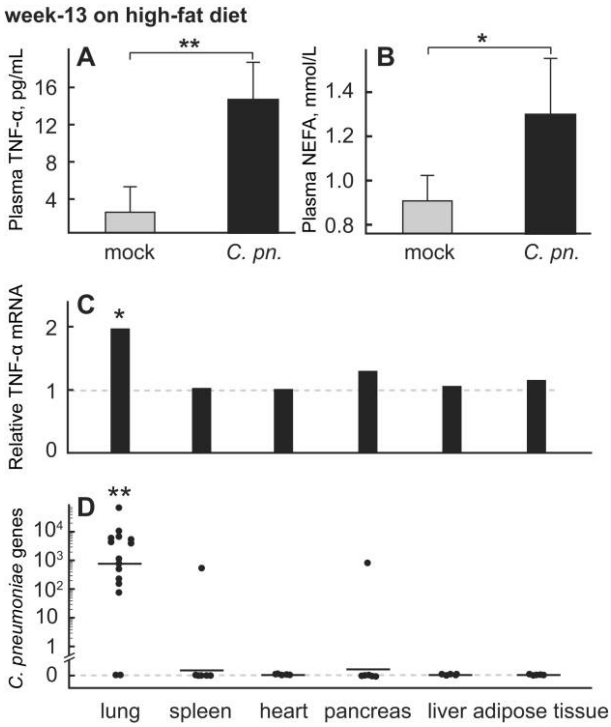

Figure 4. Infected obese B6 mice have elevated circulating tumor necrosis factor (TNF)–α levels induced by *Chlamydia pneumoniae* lung infection. *A* and *B, C. pneumoniae*–inoculated obese B6 mice (*C. pn.*) in week 13 had significantly higher levels of TNF-α (14.51 vs. 2.32 pg/mL) and nonesterified fatty acids (NEFA) (1.30 vs. 0.91 mmol/L), compared with mock-inoculated (mock) mice. The error bars represent 95% confidence intervals. *C,* In week 13, infected obese B6 mice exhibited a significant ~2-fold increase in the level of TNF-α transcripts in the lung (the primary inoculation site) but not in spleen, heart, pancreas, liver, or adipose tissue, compared with mock-infected mice. *D, C. pneumoniae* genomes per 100 mg of tissue from infected mice are plotted, and the bar indicates the mean value. Lungs from 13 of 15 infected mice had detectable *C. pneumoniae,* whereas *C. pneumoniae* was undetectable or at very low levels in other organs of infected mice. There were 15 mice in each group. $^{*}P < .05$; $^{**}P < .001$.

these tissues (figure 4*D*). These data strongly suggest that the source of increased plasma TNF-α levels is the lung, as the primary target of the intranasal *C. pneumoniae* challenge, but not organs such as liver or adipose tissue that may be secondarily infected after dispersal of *C. pneumoniae.*

TNF-α blockade or antibiotic retardation of* C. pneumoniae *prevents infection-induced insulin resistance exacerbation despite substantial bacterial dispersal. A reversal experiment tested the hypothesis that the infection-induced insulin resistance increase was mediated by TNF-α that originates from the high-level *C. pneumoniae* infection of the lung (figure 5*A*). Blockade by anti–TNF-α antibody queried the TNF-α–dependent mechanism. Treatment of obese B6 mice for 3 weeks with anti–TNF-α antibody, beginning at *C. pneumoniae* rechallenge in week 11, prevented the increase in glucose area under the curve (AUC) (figure 5*B*), insulin AUC (figure 5*C*), and insulin resistance (figure 5*D*) and reduced the increase in plasma TNF-α levels to below baseline level (figure 5*E*).

Suppression by azithromycin further tested whether high bacterial loads in the lung mediated the insulin resistance exacerbation via circulating TNF- α, or if low loads of dispersed *C. pneumoniae* organism mediated the exacerbation through action in secondary tissues. Similar to anti–TNF-α antibody, administration of azithromycin to *C. pneumoniae*–infected obese B6 mice abolished the increase in glucose AUC (figure 5*B*), insulin AUC (figure 5*C*), insulin resistance (figure 5*D*), and plasma TNF-α levels (figure 5*E*). Antibiotic therapy of chlamydial infections is bacteriostatic and is well known to induce aberrant, stalled intracellular chlamydial forms but to not eliminate the bacteria [38]. Consequently, 14 of 15 azith-

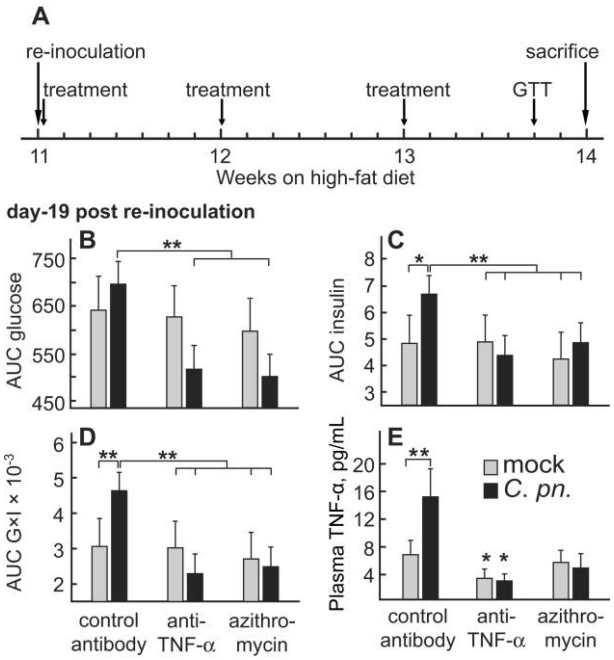

Figure 5. Tumor necrosis factor (TNF)–α blockade or antibiotic suppression of *Chlamydia pneumoniae* prevents infection-enhanced insulin resistance and increased plasma TNF-α levels. *A,* Beginning at rechallenge in week 11, mock- or *C. pneumoniae*–inoculated obese B6 mice received 3 weekly administrations of control antibody or anti–TNF-α antibody or azithromycin. Intraperitoneal glucose tolerance test (GTT) was performed 19 days after reinoculation, and mice were sacrificed 2 days later for plasma TNF-α assays. Infected obese B6 control mice demonstrated significantly higher area under the plasma concentration curve (AUC) for glucose *(B),* AUC for insulin *(C),* AUC for index for insulin resistance (G×I) (calculated as the product of fasting whole blood glucose levels (μg/L) and plasma insulin (ng/mL) concentrations [20]) *(D),* and plasma TNF-α levels *(E),* compared with infected B6 mice treated with anti–TNF-α antibody or azithromycin. There were 15 mice in each group. The error bars represent 95% confidence intervals. $^{*}P < .05$; $^{**}P < .01$.

romycin-treated mice did not eliminate *C. pneumoniae* from the lungs, even 3 weeks after challenge, but contained substantially lower bacterial loads in the lungs (42 genomes per 100 mg) than did control mice (219 genomes per 100 mg) or anti–TNF-α antibody–treated mice (3090 genomes per 100 mg) (figure 6*A*). Interestingly, significant *C. pneumoniae* dispersal to heart and adipose tissue was observed in azithromycin- and anti–TNF-α antibody–treated challenged mice (figure 6*B* and 6*C*) but not in untreated challenged control mice. However, the infection-mediated insulin resistance increase was abolished in the azithromycin- and anti–TNF-α antibody–treated mice, whereas untreated *C. pneumoniae*–challenged obese B6 mice showed increased insulin resistance.

We further corroborated the concept that circulating TNF-α emanating from the acute *C. pneumoniae* infection of the lung mediates the increased insulin resistance in the reversal experiment. Quantitative real-time PCR demonstrated significantly higher TNF-α transcript concentrations in lungs but not in heart or adipose tissue of control and anti–TNF-α antibody–treated mice, compared with azithromycin-treated mice (figure 6*D* and 6*F*). The increased in TNF-α transcripts in the lung correlated significantly with *C. pneumoniae* load in the lung ($P = .007$; $r^2 = 0.18$). In contrast, levels of TNF-α transcripts tissue were not elevated in heart and adipose tissue of mice in which low *C. pneumoniae* loads were present and did not exceed baseline transcript concentrations of uninfected tissues. These results strongly indicate that the systemic response of the acute *C. pneumoniae* infection of the lung as the primary target organ, rather than a local effect of bacteria disseminated to secondary infection sites, causes elevated levels of circulating inflammatory mediators and the infection-exacerbated insulin resistance. Thus, transient *C. pneumoniae* infection precipitates persistent and long-term enhancement of insulin resistance only in obese B6 mice, essentially amounting to a host-restricted and nutritionally restricted "hit-and-run" pathogenic mechanism.

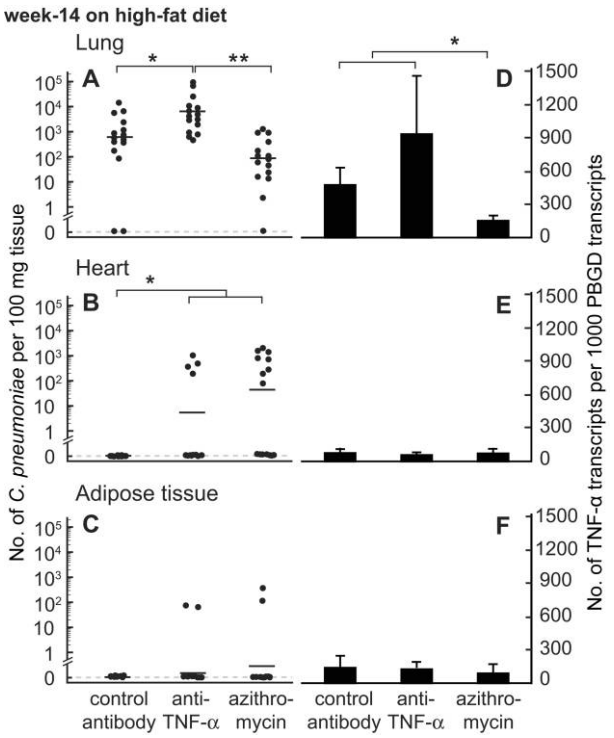

Figure 6. Mice that received azithromycin or anti–tumor necrosis factor (TNF)–α antibody demonstrated *Chlamydia pneumoniae* dissemination to heart and adipose tissue. *A,* Twenty-one days after reinoculation, *C. pneumoniae* was detected in the lungs of most infected obese B6 mice. Bacterial loads in the lungs of mice that received control antibody were significantly lower than that in mice treated with anti–TNF-α antibody but higher than that in mice treated with azithromycin. *B, C. pneumoniae* DNA was detected in heart muscle tissue of 4 infected mice treated with anti–TNF-α antibody and 8 mice treated with azithromycin but was not detectable in the hearts of infected mice that received control antibody. *C, C. pneumoniae* DNA was detected in 2 adipose tissue samples; both samples were from infected mice treated with anti–TNF-α antibody and azithromycin. *D–F,* Azithromycin-treated mice showed significantly lower TNF-α messenger RNA concentrations in lungs but not in heart or adipose tissue than did mice that received control antibody or anti–TNF-α antibody. *C. pneumoniae* loads correlated significantly with TNF-α messenger RNA levels in lungs ($P = .007$; $r^2 = 0.18$) but not in other organs. There were 15 mice in each group. The error bars represent 95% confidence intervals. *$P < .05$; **$P < .01$. PBGD, porphobilinogen deaminase.

DISCUSSION

We report here for the first time, to our knowledge, the reproduction of the causal effect of infection on insulin resistance development in an animal model. This multivariate mouse model unequivocally demonstrates a genetically and nutritionally restricted exacerbation of insulin resistance and type 2 diabetes by *C. pneumoniae* infection. Two weeks after *C. pneumoniae* reinfection, obese B6 mice exhibited higher plasma TNF-α levels and increased insulin resistance, compared with control mice (figures 1, 2, and 4*A*). Insulin sensitivity of these mice was restored to baseline by TNF-α blockade (figure 6). This confirmed that *C. pneumoniae* exacerbates insulin resistance via the noninfectious and obesity-dependent insulin resistance mechanism and that TNF-α is one of the key mediators that drive the exacerbation, similar to TNF-α released from adipose tissue [1–4, 39]. The *C. pneumoniae*–induced insulin resistance increase in obese B6 mice persisted long after elimination of *C. pneumoniae* until the end of observation period, 15-weeks after *C. pneumoniae* rechallenge (figure 3*A*). Adipose tissue of these mice showed significantly elevated levels of TNF-α, F4/80, and MCP-1 transcripts (figure 3*B*). These data are consistent with the concept of insulin resistance development and progression toward type 2 diabetes in obese mouse models that involves macrophage infiltration and overexpression of proinflammatory molecules in adipose tissue [2–4].

Infected obese B6 mice also showed significantly elevated

nonesterified fatty acid levels (figure 4*B*), which are considered to be the single most critical factor in modulating insulin sensitivity [40]. In metabolic syndrome, nonesterified fatty acids are thought to precipitate a feed-forward vicious cycle of increased insulin resistance, to impair pancreatic islet β-cell function, to reduce insulin release, and to ultimately result in type 2 diabetes. TNF-α produced for several weeks in response to the *C. pneumoniae* lung infection may have fed into the obesity-driven mechanism by promoting lipolysis [40, 41] and increased nonesterified fatty acid levels, thereby enhancing this vicious cycle and causing permanently increased insulin resistance in the absence of *C. pneumoniae* organisms.

Next, we asked whether TNF-α is mainly produced in the lung in response to the initial infection or in metabolically critical secondary organs, such as adipose tissue and liver, in response to disseminated *C. pneumoniae* organisms. Only samples from lungs demonstrated both large *C. pneumoniae* loads and high TNF-α transcript levels (figures 4*C* and 6*A*–6*C*), whereas secondary organs were essentially free of chlamydiae and had significantly lower TNF-α transcript levels (figures 4*D* and 6*D*–6*F*). With the administration of anti–TNF-α antibody and, surprisingly, azithromycin, *C. pneumoniae* disseminated significantly to the heart and adipose tissue (figure 6*B* and 6*C*), but nevertheless, insulin resistance decreased significantly to baseline levels with both treatments (figure 5*B*–5*D*). The lung as the source of insulin resistance–inducing circulating TNF-α was further verified in the reversal experiment, which demonstrated a significant correlation between large *C. pneumoniae* loads and highly up-regulated TNF-α mRNA expression in the lung ($r^2 = 0.18$; $P = .007$) but not in secondary organs (figure 6).

Our mouse model indicates that *C. pneumoniae* organisms dispersed to secondary tissues are irrelevant to insulin resistance progression and the early onset of type 2 diabetes. Rather, acute infection of the lung, the primary target of *C. pneumoniae,* causes an increase in circulating cytokines that drive the long-term insulin resistance exacerbation and accelerate type 2 diabetes onset. The concept of circulating inflammatory mediators released from the main infection site is consistent with insulin resistance–inducing mechanisms proposed for other prevalent pathogens that typically reside only at the predilection infection sites. For instance, life-long gastric infection with *Helicobacter pylori* affects 30% of the population in the United States and 80% in developing countries, causes peptic ulcer and gastric cancer, and also contributes to insulin resistance by modulating inflammatory glucose and lipid plasma profiles [10]. Similarly, ~75% and ~35% of adults in the United States have gingivitis and periodontitis, respectively, and accumulating evidence indicates that the periodontal bacterium, *Porphyromonas gingivalis,* also causes increased insulin resistance and type 2 diabetes by elevating circulating inflammatory mediators, such as TNF-α, interleukin (IL)–6, and C-reactive protein [11]. Hepatitis C virus chronically infects ~200 million individuals worldwide, and both human studies and animal models indicate that hepatitis C virus infection increases levels of circulating IL-1, IL-6, and TNF-α and the risk for insulin resistance and type 2 diabetes [12, 13]. When a combined rate of exposure to multiple pathogens is used, the epidemiological link between chronic infection and insulin resistance, as well as atherosclerosis, becomes even stronger [16, 24].

In contrast to this concept of circulating inflammatory mediators, the current perception for the involvement of *C. pneumoniae* in chronic inflammatory diseases, exemplified in coronary artery disease, stipulates that *C. pneumoniae*–infected macrophages disseminate to secondary organs and exacerbate pathological processes [7]. On the basis of the current perception, periodic antibiotic therapy would reduce cardiovascular events by excluding *C. pneumoniae* as an etiological component in atherosclerosis. However, the consistent failure to achieve this goal requires reformulating the current concept about the mechanisms of *C. pneumoniae* involvement in chronic inflammatory diseases, such as metabolic syndrome and coronary artery disease. A possible explanation of this failure is that antibiotics suppress but do not eliminate *C. pneumoniae* infection, as shown in this and other studies [38]. Could it be, however, that disseminated low-level infection is largely irrelevant to the clinical endpoint of coronary artery disease, similar to the irrelevancy of secondary organ dispersal of *C. pneumoniae* on the progression of insulin resistance in this investigation? Prospective intervention trials that failed to show beneficial effects of antibiotic administration mainly evaluated secondary coronary artery disease events [27, 28], and thus, attempted to modify the predominantly genetically and lifestyle-determined, essentially unmodifiable, terminal stage of coronary artery disease. Trials that demonstrated some antibiotic protection showed early short-term effects on coronary artery disease or reduced progression of vascular disease in patients with high *C. pneumoniae* antibody levels [25, 42–46]. Because these antibiotics are effective at suppressing acute *C. pneumoniae* infection, we propose that these protective outcomes may be related to suppression of episodes of acute reinfection [30]. Thus, consistent with the *C. pneumoniae* effect on progression of insulin resistance in this investigation, the influence of *C. pneumoniae* infection would be mediated by circulating inflammatory mediators and would largely affect the modifiable early stage of coronary artery disease progression. This concept would also be consistent with findings for other *Chlamydia*-associated diseases, such as asthma and chronic obstructive pulmonary disease, against which antichlamydial antimicrobial therapy is effective only in the early stages [47, 48].

In conclusion, this mouse model has for the first time, to our knowledge, established a causal role of infection on the

development of insulin resistance and type 2 diabetes and may be useful in the study of *C. pneumoniae* vaccination for type 2 diabetes control. However, rodent models of insulin resistance and type 2 diabetes do not reflect all aspects of human disease, and clinical studies are required for confirmation. Nevertheless, it is easy to envision that, similar to the murine model, circulating inflammatory molecules or humoral and cellular immune mediators also mediate this effect in humans, analogous to mechanisms proposed for insulin resistance enhancement by other infectious agents. Continuous, long-term antibiotic suppression of *C. pneumoniae* infection beginning early in life may be an effective but unrealistic approach to prevent *C. pneumoniae*–induced exacerbation of insulin resistance and its associated complications. Thus, vaccination may be an attractive alternative to antibiotic prophylaxis to control *C. pneumoniae*–induced exacerbation of metabolic syndrome.

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References

1. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance. Science **1993**; 259:87–91.
2. Xu H, Barnes GT, Yang Q, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest **2003**; 112:1821–30.
3. Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. Nature **2006**; 444:847–53.
4. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest **2003**; 112:1796–808.
5. Huang J, DeGraves FJ, Lenz SD, et al. The quantity of nitric oxide released by macrophages regulates *Chlamydia*-induced disease. Proc Natl Acad Sci U S A **2002**; 99:3914–9.
6. Nieto FJ. Infections and atherosclerosis: new clues from an old hypothesis? Am J Epidemiol **1998**; 148:937–48.
7. Campbell LA, Kuo CC. *Chlamydia pneumoniae*—an infectious risk factor for atherosclerosis? Nat Rev Microbiol **2004**; 2:23–32.
8. Kuo C, Campbell LA. Detection of *Chlamydia pneumoniae* in arterial tissues. J Infect Dis **2000**; 181(Suppl 3):S432–6.
9. Saikku P, Leinonen M, Mattila K, et al. Serological evidence of an association of a novel *Chlamydia*, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet **1988**; 2(8618):983–6.
10. Aydemir S, Bayraktaroglu T, Sert M, et al. The effect of *Helicobacter pylori* on insulin resistance. Dig Dis Sci **2005**; 50:2090–3.
11. Mealey BL, Rose LF. Diabetes mellitus and inflammatory periodontal diseases. Curr Opin Endocrinol Diabetes Obes **2008**; 15:135–41.
12. Narita R, Abe S, Kihara Y, Akiyama T, Tabaru A, Otsuki M. Insulin resistance and insulin secretion in chronic hepatitis C virus infection. J Hepatol **2004**; 41:132–8.
13. Shintani Y, Fujie H, Miyoshi H, et al. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. Gastroenterology **2004**; 126:840–8.
14. Aboud M, Elgalib A, Kulasegaram R, Peters B. Insulin resistance and HIV infection: a review. Int J Clin Pract **2007**; 61:463–72.
15. Madjid M, Awan I, Ali M, Frazier L, Casscells W. Influenza and atherosclerosis: vaccination for cardiovascular disease prevention. Expert Opin Biol Ther **2005**; 5:91–6.
16. Espinola-Klein C, Rupprecht HJ, Blankenberg S, et al. Impact of infectious burden on progression of carotid atherosclerosis. Stroke **2002**; 33:2581–6.
17. Kuo CC, Jackson LA, Campbell LA, Grayston JT. *Chlamydia pneumoniae* (TWAR). Clin Microbiol Rev **1995**; 8:451–61.
18. Boman J, Söderberg S, Forsberg J, et al. High prevalence of *Chlamydia pneumoniae* DNA in peripheral blood mononuclear cells in patients with cardiovascular disease and in middle-aged blood donors. J Infect Dis **1998**; 178:274–7.
19. Smieja M, Mahony J, Petrich A, Boman J, Chernesky M. Association of circulating *Chlamydia pneumoniae* DNA with cardiovascular disease: a systematic review. BMC Infect Dis **2002**; 2:21.
20. Nabipour I, Vahdat K, Jafari SM, Pazoki R, Sanjdideh Z. The association of metabolic syndrome and *Chlamydia pneumoniae*, *Helicobacter pylori*, cytomegalovirus, and herpes simplex virus type 1: the Persian Gulf Healthy Heart Study. Cardiovasc Diabetol **2006**; 5:25.
21. Ekesbo R, Nilsson PM, Lindholm LH, Persson K, Wadström T. Combined seropositivity for *H. pylori* and *C. pneumoniae* is associated with age, obesity and social factors. J Cardiovasc Risk **2000**; 7:191–5.
22. Campbell LA, Moazed TC, Kuo CC, Grayston JT. Preclinical models for *Chlamydia pneumoniae* and cardiovascular disease: hypercholesterolemic mice. Clin Microbiol Infect **1998**; 4(Suppl 4):S23–S32.
23. Dart AM, Martin JL, Kay S. Association between past infection with *Chlamydia pneumoniae* and body mass index, low-density lipoprotein particle size and fasting insulin. Int J Obes Relat Metab Disord **2002**;26: 464–8.
24. Fernández-Real JM, López-Bermejo A, Vendrell J, Ferri MJ, Recasens M, Ricart W. Burden of infection and insulin resistance in healthy middle-aged men. Diabetes Care **2006**; 29:1058–64.
25. Gupta S, Leatham EW, Carrington D, Mendall MA, Kaski JC, Camm AJ. Elevated *Chlamydia pneumoniae* antibodies, cardiovascular events, and azithromycin in male survivors of myocardial infarction. Circulation **1997**; 96:404–7.
26 Falck G, Gnarpe J, Hansson LO, Svärdsudd K, Gnarpe H. Comparison of individuals with and without specific IgA antibodies to *Chlamydia pneumoniae*: respiratory morbidity and the metabolic syndrome. Chest **2002**; 122:1510–2.
27. Cannon CP, Braunwald E, McCabe CH, et al. Antibiotic treatment of *Chlamydia pneumoniae* after acute coronary syndrome. N Engl J Med **2005**; 352:1646–54.
28. Grayston JT, Kronmal RA, Jackson LA, et al. Azithromycin for the secondary prevention of coronary events. N Engl J Med **2005**; 352: 1637–45.
29. O'Connor CM, Dunne MW, Pfeffer MA, et al. Azithromycin for the secondary prevention of coronary heart disease events: the WIZARD study: a randomized controlled trial. JAMA **2003**; 290:1459–66.
30. Muhlestein JB. Secondary prevention of coronary artery disease with antimicrobials: current status and future directions. Am J Cardiovasc Drugs **2002**; 2:107–18.
31. Duncan MH, Singh BM, Wise PH, Carter G, Alaghband-Zadeh J. A simple measure of insulin resistance. Lancet **1995**; 346:120–1.
32. Yang Q, Graham TE, Mody N, et al. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature **2005**; 436:356–62.
33. Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, Feinglos MN. Diet-induced type II diabetes in C57BL/6J mice. Diabetes **1988**; 37:1163–7.
34. Wang C, van Ginkel FW, Kim T, et al. Temporal delay of peak T cell immunity determines *Chlamydia pneumoniae* pulmonary disease in mice. Infect Immun **2008**; 76:4913–23.
35. Stevenson MM, Kongshavn PA, Skamene E. Genetic linkage of resistance to *Listeria monocytogenes* with macrophage inflammatory responses. J Immunol **1981**; 127:402–7.

36. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. Proc Natl Acad Sci U S A **2003**; 100:7265–70.
37. Campbell LA, Nosaka T, Rosenfeld ME, Yaraei K, Kuo CC. Tumor necrosis factor alpha plays a role in the acceleration of atherosclerosis by *Chlamydia pneumoniae* in mice. Infect Immun **2005**; 73:3164–5.
38. Gieffers J, Füllgraf H, Jahn J, et al. *Chlamydia pneumoniae* infection in circulating human monocytes is refractory to antibiotic treatment. Circulation **2001**; 103:351–6.
39. Feinstein R, Kanety H, Papa MZ, Lunenfeld B, Karasik A. Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. J Biol Chem **1993**; 268: 26055–8.
40. Laurencikiene J, van Harmelen V, Arvidsson Nordström E, et al. NF-κB is important for TNF-α-induced lipolysis in human adipocytes. J Lipid Res **2007**; 48:1069–77.
41. Souza SC, Palmer HJ, Kang YH, et al. TNF-alpha induction of lipolysis is mediated through activation of the extracellular signal related kinase pathway in 3T3-L1 adipocytes. J Cell Biochem **2003**; 89:1077–86.
42. Gurfinkel E, Bozovich G, Beck E, Testa E, Livellara B, Mautner B. Treatment with the antibiotic roxithromycin in patients with acute non-Q-wave coronary syndromes. The final report of the ROXIS Study. Eur Heart J **1999**; 20:121–7.
43. Sander D, Winbeck K, Klingelhöfer J, Etgen T, Conrad B. Reduced progression of early carotid atherosclerosis after antibiotic treatment and *Chlamydia pneumoniae* seropositivity. Circulation **2002**; 106:2428–33.
44. Neumann F, Kastrati A, Miethke T, et al. Treatment of *Chlamydia pneumoniae* infection with roxithromycin and effect on neointima proliferation after coronary stent placement (ISAR-3): a randomised, double-blind, placebo-controlled trial. Lancet **2001**; 357:2085–9.
45. Sander D, Winbeck K, Klingelhöfer J, Etgen T, Conrad B. Reduced progression of early carotid atherosclerosis after antibiotic treatment and *Chlamydia pneumoniae* seropositivity. Circulation **2002**; 106:2428–33.
46. Jespersen CM, Als-Nielsen B, Damgaard M, et al. Randomised placebo controlled multicentre trial to assess short term clarithromycin for patients with stable coronary heart disease: CLARICOR trial. BMJ **2006**; 332:22–7.
47. Hahn DL. *Chlamydia pneumoniae*, asthma, and COPD: what is the evidence? Ann Allergy Asthma Immunol **1999**; 83:271–88, 291–2.
48. Karnak D, Beder S. Treatment of *Chlamydia pneumoniae* infection and chronic obstructive pulmonary disease. Expert Opin Pharmacother **2002**; 3:1461–70.