Apolipoprotein E–Deficient Mice Develop an Anti–Chlamydophila pneumoniae T Helper 2 Response and Resist Vascular Infection

Dani Nazzal, Nicole Therville, Houda Yacoub-Youssef, Virginie Garcia, Mogens Thomsen, Thierry Levade, Bruno Segui, and Hervé Benoist

Institut National de la Santé et de la Recherche Médicale Unité 858, Institute of Molecular Medicine of Rangueil, Institut Fédératif de Recherche F150, and University Paul Sabatier, Toulouse, France

Background. Hypercholesterolemia could inhibit the immune response against various pathogens. No information is available about its impact on the immune response toward Chlamydophila pneumoniae.

Methods. Apolipoprotein E (apoE)–deficient and wild-type mice fed a normal diet were infected with a single intranasal inoculation of viable C. pneumoniae.

Results. Whereas interferon γ concentrations (T helper 1 response) were similar in the lungs and spleen of apoE-deficient and wild-type mice, increased concentrations of interleukin 10, interleukin 6, and interleukin 4 (T helper 2 response) were found in the lungs of apoE-deficient mice. The spleen B lymphocyte percentage and interleukin 4 levels and serum specific antibody titers were higher in apoE-deficient mice.

C. pneumoniae infection was facilitated neither in the lungs nor in the aorta of these mice. On the contrary, the number of apoE-deficient mice with detectable levels of bacterial DNA in the aorta was clearly decreased. When low-density lipoprotein receptor–deficient mice fed a normal diet were similarly infected, no difference in the interleukin 4 concentration and infection level was observed in the lungs and no protection was found in the aorta.

Conclusions. Mild hypercholesterolemia in mice does not facilitate C. pneumoniae persistence in the vascular wall. ApoE deficiency, rather than mild hypercholesterolemia, probably favors the development of an unusual anti–C. pneumoniae T helper 2 response and protects against vascular infection.

Chlamydia pneumoniae is a gram-negative pathogen that can persist in the body and might be involved in chronic inflammatory diseases such as asthma, multiple sclerosis, reactive arthritis, and atherosclerosis [1]. The protective immune response against C. pneumoniae is quite well characterized. Studies of mouse models have shown that interferon γ (IFN-γ)–dependent immune responses are critical for effective protection [2, 3]. However, although the mechanisms may differ between humans and mice (eg, at the level of indoleamine 2,3-dioxygenase induction [4, 5]), low levels of IFN-γ facilitate the induction of intracellular C. pneumoniae persistence in vitro, which suggests a dual role for this cytokine in vivo [6]. Like other pathogens, C. pneumoniae could employ various mechanisms to evade the host’s immune response, such as induction of interleukin 10 (IL-10) secretion [7], inhibition of antigen presentation [8], or resistance to apoptosis [9].

To our knowledge, no information is available about the impact of hypercholesterolemia on the immune response toward C. pneumoniae. Hypercholesterolemia may favor, at least partly, C. pneumoniae growth. Indeed, several cell culture experiments demonstrated that intracellular growth of Chlamydia species requires host cell lipids, such as cholesterol [10]. C. pneumoniae–specific T cells that expressed cytotoxic function and a T helper 1 (Th1) cytokine profile were detected in the atherosclerotic lesions of patients with antibodies against C. pneumoniae [11]. The persistence of C. pneumoniae infection in the vascular wall of patients with atherosclerosis suggests that the immune protection against the bacteria is altered. On one hand, C. pneumoniae might...
participate in the disease process [12], promoting inflammation [1, 12–14]. However, in studies with hypercholesterolemic apolipoprotein E (apoE)–deficient mice, *C. pneumoniae* infection did not always facilitate atherosclerosis [15, 16]. On the other hand, a dysregulated immune function related to hypercholesterolemia and/or lipid oxidation might facilitate infection [17, 18]. Indeed, hypercholesterolemia can inhibit the immune response against various infectious agents in apoE-deficient or low-density lipoprotein receptor (LDLR)–deficient mice [19–23].

The aim of the present study was to investigate the susceptibility to infection and the immune response of hypercholesterolemic mice that were intranasally infected with *C. pneumoniae*. We found that the immune response against the bacteria was altered in apoE-deficient mice, without compromising bacterial elimination, neither in the lungs nor in the vascular wall.

**METHODS**

*C. pneumoniae* strain and inoculum preparation. The TW183 *C. pneumoniae* strain was purchased from the American Type Culture Collection (ATCC) and was propagated on HEp-2 cells (ATCC), titered (as infection-forming units [IFUs] per milliliter), and stored at −70°C until use, as described elsewhere [7].

Infection and immunization of mice. Twelve- to sixteen-week-old male C57BL/6 wild-type (WT) mice, apoE-deficient mice, and LDLR-deficient mice (Iffa Credo) were fed a regular mouse chow diet ad libitum (all strains were on a C57BL/6 background). After mild anesthesia (250-μL intraperitoneal injection of 5 mg/mL ketamine), mice were inoculated intranasally with *C. pneumoniae*. We found that the immune response against the bacteria was altered in apoE-deficient mice, without compromising bacterial elimination, neither in the lungs nor in the vascular wall.

**Flow cytometric analysis of infected mouse splenocytes.** In uninfected mice, the weights of the spleens and the numbers of splenocytes were 25% greater in apoE-deficient mice than in age-matched WT controls when normalized to body weight [18] (D. Nazzal et al, unpublished data, 2009). Consequently, only the percentage of B and T lymphocytes was evaluated in infected mouse spleens. Isolated spleen suspensions were incubated in triplicate on ice with fluorescent anti-CD4, anti-CD8, anti-CD19 (Caltag), anti-CD1d, anti-CD43, and anti-CD5 (PharMingen) monoclonal antibodies, then analyzed by flow cytometry with CellQuest Pro software (version 4.0.1; Becton Dickinson).

Assessment for cytokine detection after in vitro specific activation by *C. pneumoniae*. Splenocytes (10^5 cells/mL in RPMI 1640 medium with 10% fetal calf serum [Eurobio]), nonessential amino acids (0.1 mmol/L), sodium pyruvate (1 mmol/L), 1-glutamine (100 mmol/L), and β-mercaptoethanol (20 μmol/L) were incubated with heat-inactivated elementary bodies (10^5 IFUs/mL) of *C. pneumoniae*, mixed with 10^6 cells/mL irradiated WT splenocytes (3000 rad/min). After 96 h of incubation, supernatants were replaced by fresh medium containing interleukin 2 (50 IU/mL) for an additional 48 h. Supernatants were collected on days 3, 4, and 6 and assayed for cytokine content. In some experiments, the proliferative response was measured by incorporation of tritium-labeled thymidine over the past 24 h of incubation.

Analysis of humoral responses and serum cholesterol level. *C. pneumoniae*–specific antibodies (immunoglobulin G [IgG] plus immunoglobulin M [IgM] and IgM) were detected in serum samples by use of a microimmunofluorescence assay (Organium). The antibody titer was defined as the last serum dilution that gave positive fluorescence. The serum total cholesterol concentration was determined by an enzymatic method using reagents from Oriiba ABX.

Immunohistochemistry. Lungs were removed and frozen in liquid nitrogen. Serial 7-μm-thick sections were prepared, air-dried, and fixed in acetone. Endogenous biotin and peroxi
dases were blocked using a biotin-blocking system and a peroxidase-blocking reagent (Dako). The sections were stained with rat antimouse CD45, CD4, CD8, and CD19 (PharMingen), incubated with biotinylated goat antirat antibody (PharMingen) and streptavidin-peroxidase (ABCcomplex/horseradish peroxidase kit; Dako), and then counterstained with hematoxylin.

The degree of inflammation was evaluated by 2 independent observers using anti-CD45 staining and at least 2 sections per mouse, and then the mean inflammation score was calculated per mouse with the use of the classical immunofluorescence assay with HEp-2 cells and a fluorescein isothiocyanate–conjugated anti–*C. pneumoniae* monoclonal antibody (Biorad). Bacterial titers were expressed as IFUs per milligram of lung protein.
Figure 1. Infection and cytokine levels in lungs after Chlamydia pneumoniae (CP) inoculation in apolipoprotein E (apoE)-deficient (apoE−/−) mice. In 4 separate experiments, wild-type (wt; n = 22) and apoE-deficient (n = 22) mice were inoculated intranasally with C. pneumoniae (5 × 10^7 infection-forming units [IFUs]). Eighteen days after inoculation, homogenized lung supernatants were used to determine C. pneumoniae titers (n = 22 for each group) (A) and interleukin 10 (IL-10), interleukin 6 (IL-6), interleukin 4 (IL-4), and interferon γ (IFN-γ) concentrations (representative of 2 experiments; n = 6 uninfected mice; n = 10–12 infected mice) (B). Data are shown as the mean ± standard deviation. *P < .05.

as follows: 0, no inflammation; 1, mild inflammation; 2, moderate inflammation; or 3, strong inflammation. The number of infiltrates that tested positive for CD19-positive, CD4-positive, or CD8-positive lymphocytes was evaluated.

Qualitative polymerase chain reaction (PCR) analysis of C. pneumoniae infection. Aliquots of lung and aorta specimens (about 80 mg of fresh tissue) were suspended in 0.25 mL of lysis buffer (PureLink Quick Plasmid Miniprep kit; Invitrogen/Gibco) by use of a FastPrep instrument (MP-Biomedicals). DNA was extracted using phenol-chloroform. Amplification of C. pneumoniae DNA was performed as reported elsewhere [24], by use of 1 U of GoTaq Flexi DNA polymerase (Promega) in a total volume of 25 μL. PCR products were separated by electrophoresis on 1% agarose gels and visualized after staining with Sybr Safe (Invitrogen). By following this protocol, as little as 0.3 pg of C. pneumoniae DNA could be detected.

Statistical analysis. Data are represented as the mean (± standard deviation [SD] or ± standard error of the mean). Mean values were compared using the Student t test. Differences were considered significant when P < .05.

RESULTS

Increased concentrations of T helper 2 (Th2) cytokines in lungs of C. pneumonia–infected apoE-deficient mice do not affect the elimination of local bacteria. Pulmonary infection in C57BL/6 mice has been shown to be maximal at 10–12 d after infection and always detectable on day 30 after a single intranasal inoculation [25]. Eighteen days after a single intranasal inoculation, viable C. pneumoniae bacilli were detected in the lungs of 19 of 22 infected apoE-deficient mice, compared with 22 of 22 infected WT mice, which corresponds to the decrease and resolving phase of lung infection. The level of residual lung infection was slightly but not statistically significantly reduced in apoE-deficient mice (Figure 1A). The lung IL-10 concentration was similar in infected and uninfected WT mice, whereas a sta-
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Figure 2. Histological comparison of leukocyte infiltration in lungs. Wild-type (wt) and apolipoprotein E (apoE)–deficient (apoE−/−) mice were intranasally infected (plus signs) with Chlamydia pneumoniae (CP) or left uninfected (minus signs). Eighteen days after inoculation, lung sections were treated with a rat antimouse CD45 monoclonal antibody, revealed with peroxidase staining, or untreated (control [cont]), then colored with hematoxylin (original magnification, ×250).
Figure 3. Specific antibodies in serum, cytokine production, and lymphocytes in the spleen of *Chlamydia pneumoniae* (CP)–infected apolipoprotein E (apoE)–deficient (apoE−/−) mice. ApoE-deficient and wild-type (wt) mice were infected intranasally with *C. pneumoniae*. Eighteen days after inoculation, spleens and serum samples were harvested. Splenocytes were incubated with heat-inactivated *C. pneumoniae* elementary bodies; after 3, 4, or 6 d, cytokine concentrations were determined by enzyme-linked immunosorbent assay. Data are shown as the mean (± standard deviation [SD]) of 10 infected mice (representative of 2 experiments) (A). Spleen cells were stained with anti-CD19, anti-CD4, or anti-CD8 antibodies and then analyzed by flow cytometry. Data are shown as the mean (±SD) of the number of mice shown in parentheses (results of 4 independent experiments); *P<.05 (B). *C. pneumoniae*–specific serum antibodies were measured in serum samples of infected mice. Data are shown as the mean (±SD) of 22 infected mice; *P<.05 for the difference between wild-type and apoE-deficient mice (C). IFN-γ, interferon γ; IgG, immunoglobulin G; IgM, immunoglobulin M; IL-4, interleukin 4.

Spleen lymphocyte populations showed that the percentage of CD19-positive cells was increased in infected apoE-deficient mice, compared with infected WT mice (Figure 3B). The percentages of CD4-positive and CD8-positive T cells were lower in infected apoE-deficient mice than in infected WT mice; this was also observed for CD8-positive lymphocytes, compared with uninfected apoE-deficient mice (Figure 3B). This relative decrease of the T cell percentage could be, at least partly, a consequence of facilitation of B cell expansion and/or T cell development inhibition in infected apoE-deficient mice, compatible with an increased antibody response. Finally, in *C. pneumoniae*–infected apoE-deficient mice, both IgM-specific and IgM+IgG-specific antibody titers in the serum samples were higher than those of WT mice, which suggests that the development of a systemic antibody response against *C. pneumoniae* was altered in apoE-deficient mice (Figure 3C). Because B cells, in addition to their ability to produce antibodies, have additional functions such as the production of cytokines and the regulation of the immune response (eg, in infections [26]), different B cell subsets in the spleen were explored by means of flow cytometry 24 d after infection. Compared with infected WT mice, the percentage of B1a cells (CD19+CD43+CD5+), which are known to be a major source of IL-10 [27], as well as the percentage of regulatory B10 cells (CD19+CD5+CD1dhigh) [28], were not statistically significantly altered in apoE-deficient animals (data not shown). However, the mean (±SD) percentage of CD19+CD43+CD1dhigh cells described as marginal zone B cells [29] and also containing regulatory B cells [30] was 9.3% ± 2.4% in *C. pneumoniae*–infected apoE-deficient mice (*n* = 7 from 2 separate experiments), compared with 6.5% ± 1.9% in infected WT mice (*n* = 7; *P<.02*).

Moreover, after 4 injections of bovine serum albumin (100 µg per mouse), a thymo-dependent antigen, uninfected apoE-
deficient mice developed a high specific antibody response that was dramatically stronger than the response in WT counterparts (data not shown). These results confirm that the antibody response was facilitated in apoE-deficient mice.

**C. pneumoniae infection of the vascular wall in apoE-deficient and LDLR-deficient mice.** Because *C. pneumoniae* can be detected in the mouse aorta after intranasal inoculation [31], the consequences of the above-mentioned immunological alterations on vascular infection were investigated. Compared with WT animals, a statistically significant increase of IL-4 was always observed in the lungs of infected apoE-deficient mice 24 d after inoculation (Figure 4A), with no difference in IFN-γ concentrations (data not shown). However, lung infection was slightly, but not statistically significantly, attenuated in apoE-deficient mice, as shown by both a cellular culture detection method and PCR analysis (Figure 4A). Rather unexpectedly, PCR analysis indicated that only 1 of 7 apoE-deficient mice had detectable levels of *C. pneumoniae* DNA in the aorta, compared with 4 of 4 WT mice. Two further independent experiments showed that 1 of 3 and 1 of 4 apoE-deficient mice had detectable levels of *C. pneumoniae* DNA in the aorta, compared with 3 of 3 and 4 of 4 WT mice, 24 d after inoculation (data not shown). Taken together, the present data suggest that the immune system of apoE-deficient mice allows for efficient *C. pneumoniae* clearance 24 d after infection, both in the lungs and in the aorta.

Additional experiments were conducted using LDLR-deficient mice that were fed a normal diet. Eighteen days after inoculation, no lethality was observed in infected WT and LDLR-deficient mice. The IFN-γ concentration was equally increased in the lungs of infected LDLR-deficient and WT mice (data not shown), without a statistically significant increase in IL-4 concentration in LDLR-deficient mice compared with WT mice, which suggests that the local Th1 cytokine response is not associated with a Th2 response in LDLR-deficient mice (Figure 4B). The moderate hypercholesterolemia was associated with no statistically significant alteration of the infection, neither in the lungs nor in the vascular wall (Figure 4B). In contrast to those of apoE-deficient mice, the aortas of all LDLR-deficient mice were positive for bacterial DNA (Figure 4B).

**DISCUSSION**

When fed a normal diet, apoE-deficient mice display hypercholesterolemia and develop fatty streaks in the proximal aorta at 3 months of age, whereas atherosclerosis and fibrous plaques can appear at 5 months of age [32]. Our observations of 3–4-month-old apoE-deficient mice point to the development of

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**Figure 4.** Detection of *Chlamydia pneumoniae* in the lungs and aorta of apolipoprotein E (apoE)-deficient (apoE−/−) and low-density lipoprotein receptor (LDLR)-deficient (LDLR−/−) mice. Wild-type (wt), apoE-deficient, and LDLR-deficient mice were inoculated intranasally with *C. pneumoniae*. Before inoculation, the serum cholesterol concentration was determined and the means (± standard deviation [SD]) were calculated. Lungs and aortas were homogenized to evaluate the following: (1) the interleukin 4 (IL-4) concentration in lung supernatants [data are shown as the mean ± SD; *P < .05], (2) the lung infection using the HEp-2 cellular test (2 determinations; results are expressed in infection-forming units [IFUs] per milligram of protein as the mean ± standard error of the mean and as the ratio of the number of mice that tested positive to the total number of mice), and (3) the lung and vascular infection using polymerase chain reaction (3–5 determinations; results are expressed as the ratio of the number of mice that tested positive to the total number of mice). A, ApoE-deficient and wild-type mice, 24 d after inoculation. B, LDLR-deficient and wild-type mice, 18 d after inoculation.
an efficient immune defense against *C. pneumoniae* in the lungs 18 and 24 d after infection. In PCR analysis, bacterial DNA was found in the cardiovascular tissues of only 21% of apoE-deficient mice (*n* = 14), compared with 100% of WT mice (*n* = 11), 24 d after infection. Thus, *C. pneumoniae* infection was facilitated neither in the lungs nor in the vascular wall of apoE-deficient mice. In addition, experiments performed 18 d after infection and using the LDLR-deficient mouse model confirmed that moderate hypercholesterolemia does not favor *C. pneumoniae* vascular infection in mice fed a normal diet, at least during the 3 weeks following a single inoculation.

Several previous studies using hypercholesterolemic mice reported alterations in the immune responses toward *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Candida albicans*, lymphocytic choriomeningitis virus, and *Mycobacterium tuberculosis* [19–23]. However, except for the last study [23], all infections were performed intravenously or intraperitoneally, which bypassed the role of the surface immune system, and sometimes the mice were fed a hypercholesterolemic diet [22, 23]. The immune defense against all of these pathogens requires Th1 cytokines. The general conclusion of these observations is that there exists a hypercholesterolemia-induced impairment of IFN-γ-dependent and Th1-driven immune responses against infectious agents, at least when they are injected into the peritoneal cavity or the blood stream. Paradoxically, intranasal *C. pneumoniae* inoculation and feeding the mice a normal diet produced results suggestive of the development by control and apoE-deficient mice of an immune response with at least a similar efficiency to eliminate *C. pneumoniae* in the lungs and vascular wall. It is tempting to speculate that *C. pneumoniae* elimination could be facilitated in apoE-deficient mice. Indeed, apoE-deficient mice showing detectable levels of *C. pneumoniae* DNA in the lung and vascular wall were less numerous than their WT counterparts. In agreement with previous data that underlined the important role of IFN-γ in resistance to *C. pneumoniae* infection [33], a similar increase in IFN-γ concentration was measured in the lungs of infected WT and apoE-deficient mice, which suggests that the IFN-γ–mediated defense was unaltered in apoE-deficient mice. However, because IL-10, IL-6, and IL-4 concentrations were statistically significantly increased in infected apoE-deficient mice compared with those in infected WT mice, the quality of the local immune response could be different. IL-10 and IL-4 are known to antagonize Th1 and IFN-γ responses. Actually, IFN-γ production was not reduced in the lungs of infected apoE-deficient mice, and the high IL-10, IL-6, and IL-4 concentrations are compatible with a local Th2 response, which suggests the development of a mixed Th1/Th2-type response. Because B cells can produce various cytokines (eg, IFN-γ, IL-10, and IL-4) [34], in infected apoE-deficient mice, cytokines could be produced, at least in part, by certain subpopulations of B lymphocytes. Finally, B cells from the splenic marginal zone, which play a protective role in immunity during bloodborne bacterial infection [35], were increased in infected apoE-deficient mice—a finding compatible with vascular protection against *C. pneumoniae* infection.

The results of several recent studies have indicated that apoE protein can modulate the immune response and inflammation [36, 37]. For instance, apoE was demonstrated to suppress a Th1 response in vivo [37]. Consequently, an efficient Th1 response against the bacteria could be preserved, in spite of a high Th2 response. In addition, in apoE-deficient mice, an inhibition of the dissemination and persistence of bacteria in the vascular wall could be associated, at least partly, with a high specific antibody production that is oriented toward extracellular *C. pneumoniae*. Finally, compared with LDLR-deficient mice, the partial protection observed in apoE-deficient mice is likely a consequence of apoE deficiency rather than moderate hypercholesterolemia.

To our knowledge, only 2 studies using the apoE-deficient mouse model have proposed that hypercholesterolemia favors the antibody response (against both the altered low-density lipoprotein autoantigen and the exogenous keyhole limpet hemocyanin antigen [38] or against tetanus toxoid [18]). Similarly, our data show that the antibody response against *C. pneumoniae*, as well as against bovine serum albumin (data not shown) or alloantigens [39], is facilitated in apoE-deficient mice. In the spleen of infected apoE-deficient mice, the high ratio of IL-4–producing cells to IFN-γ–producing cells and of CD19 cells to CD4 or CD8 cells is compatible with the facilitation of a B lymphocyte response. Taken together, our findings are consistent with those in previous reports and strongly indicate that antibody responses are facilitated in apoE-deficient mice.

In agreement with the results of the present work, some studies have reported the absence of *C. pneumoniae* DNA in the aortic tissue of infected apoE-deficient mice, even after multiple infections [15]. Several factors probably modulate vascular infection, such as the age of the animals, the duration of the postinoculation period, and the quality and kinetics of the immune response [40]. The present data point to a further factor—apoE deficiency. Indeed, as explained above, it is possible that the specific Th2 response against the bacteria helps its elimination as a viable bacteria. In addition, the age of the mice used in our study is compatible with the presence of foam cells in the vascular wall, which could inhibit the growth of *C. pneumoniae* but remain sensitive to the inflammatory effects of *C. pneumoniae* antigens [41]. Indeed, proinflammatory membrane components could persist in the vascular wall for a long time in the absence of bacterial DNA and viable bacteria [42]. Therefore, in infected apoE-deficient mice, facilitation of atherogenesis could result from the combination of putative

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persistence of C. pneumoniae antigens, the exacerbated immune response due to apoE deficiency, and the inflammatory effect of hypercholesterolemia.

Acknowledgment

We thank Dr Nelly Blaes for helpful discussion and Jean Claude Thiers and Patricia Clavé for excellent technical assistance.

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