

Hypercholesterolemia Impairs Immunity to Tuberculosis[∇]

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We demonstrate that apolipoprotein E-deficient (*ApoE*^{-/-}) mice are highly susceptible to tuberculosis and that their susceptibility depends on the severity of hypercholesterolemia. Wild-type (WT) mice and *ApoE*^{-/-} mice fed a low-cholesterol (LC) or high-cholesterol (HC) diet were infected with ~50 CFU *Mycobacterium tuberculosis* Erdman by aerosol. *ApoE*^{-/-} LC mice were modestly more susceptible to tuberculosis than WT LC mice. In contrast, *ApoE*^{-/-} HC mice were extremely susceptible, as evidenced by 100% mortality after 4 weeks with tuberculosis. The lung pathology of *ApoE*^{-/-} HC mice was remarkable for giant abscess-like lesions, massive infiltration by granulocytes, elevated inflammatory cytokine production, and a mean bacterial load ~2 log units higher than that of WT HC mice. Compared to WT HC mice, the gamma interferon response of splenocytes restimulated ex vivo with *M. tuberculosis* culture filtrate protein was delayed in *ApoE*^{-/-} HC mice, and they failed to control *M. tuberculosis* growth in the lung. OT-II cells adoptively transferred into uninfected *ApoE*^{-/-} HC mice had a weak proliferative response to their antigen, indicating impaired priming of the adaptive immune response. Our studies show that *ApoE*^{-/-} deficiency is associated with delayed expression of adaptive immunity to tuberculosis caused by defective priming of the adaptive immune response and that elevated serum cholesterol is responsible for this effect.

Dyslipidemia is a growing health problem in many industrialized countries and developing nations (33). High cholesterol is one of the most common forms of dyslipidemia, with 17% of adults in the United States alone being hypercholesterolemic (15). The impact of hypercholesterolemia on inflammation and atherosclerosis has received considerable attention, but much less is known about its potential effects on protective immunity. However, limited work with hypercholesterolemic apolipoprotein E-deficient (*ApoE*^{-/-}) and low-density lipoprotein receptor deficient (*LDL-R*^{-/-}) mice has demonstrated that the effects of hypercholesterolemia on the immune system extend beyond promoting inflammation.

The LDL-R on hepatocytes removes cholesterol from the blood by binding to LDL particles via ApoE; thus, LDL-R- and ApoE-deficient mice are spontaneously hypercholesterolemic. *ApoE*^{-/-} mice were reported to have impaired defense against *Candida albicans*, *Listeria monocytogenes*, *Klebsiella pneumoniae*, and lymphocytic choriomeningitis virus (LCMV) infection (1, 5, 11, 25). Decreased resistance of *LDL-R*^{-/-} mice to LCMV has also been described (11). Immunologic disorders, including Th2 skewing of antibody responses and impaired dendritic cell (DC) trafficking, have been described in *ApoE*^{-/-} mice (2, 24, 35). The proposed mechanisms for the increased susceptibility of hypercholesterolemic mice to infectious diseases are diverse and range from increased availability of lipids as a nutrient source for microbes to detoxification of lipopolysaccharide by ApoE, reduced phagocytic capacity, and impaired cytotoxic-T-lymphocyte activation. An improved un-

derstanding of how hyperlipidemia affects immunity will allow a more complete assessment of the health risks of hyperlipidemia and the populations most at risk for infectious diseases.

The World Health Organization predicts there will be 1 billion new *Mycobacterium tuberculosis* infections, 150 million new active tuberculosis (TB) cases, and 37 million TB-related deaths by 2020 (34). About one-third of the world's population is *M. tuberculosis* infected, and of this population, ~10% will develop active TB disease. Increased risk for TB disease is based on both inherited and acquired factors. Given the large number of persons with latent TB infection, even a modest impairment of protective immunity by hyperlipidemia could have a substantial impact on the public health burden of TB.

Here, we have investigated the effect of hypercholesterolemia on TB immunity by challenging wild-type (WT) and *ApoE*^{-/-} mice with a low aerosol dose of *M. tuberculosis* Erdman. To test whether the *ApoE*^{-/-} phenotype in the TB model was a function of hypercholesterolemia and not ApoE deficiency per se, mice were fed a low-cholesterol (LC) or high-cholesterol (HC) "Western" diet. *ApoE*^{-/-} LC mice were slightly more susceptible to TB, as evidenced by increased lung inflammation and bacterial lung burden. In contrast, *ApoE*^{-/-} HC mice demonstrated extreme TB susceptibility characterized by massive lung inflammation, heavy bacterial burden, and early mortality. Their failure to mount a timely protective Th1 immune response to TB was due in part to impaired priming of the adaptive immune response.

MATERIALS AND METHODS

Animals. C57BL/6 and B6.129P2-Apoe^{tm1UncN11} (*ApoE*^{-/-}) mice were purchased from Taconic Farms or bred at the University of Massachusetts Medical School (UMMS) with breeders purchased from The Jackson Laboratory. *ApoE*^{-/-} mice were originally derived by Piedrahita et al. (23) and backcrossed on a C57BL/6 background for more than 10 generations. C57BL/6 OT-II mice were a kind gift from Kenneth Rock, UMMS. The mice were housed within the

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Animal Medicine facility at UMMS, and the Institutional Animal Care and Use Committee approved these experiments.

Reagents. The mice were fed either an LC (0.12% cholesterol) or an HC (1.25% cholesterol, 0.5% sodium cholate; Research Diets) diet 2 weeks before and during each experiment. Ovalbumin (OVA)-coated iron beads were a kind gift from Lian Jun Shen, UMMS. Chicken OVA was covalently bound to iron oxide beads according to the manufacturer's protocol (BioMag Amine; Polyscience Inc.). Culture filtrate protein (CFP) was obtained by growing *M. tuberculosis* Erdman in Sauton's broth for 1 month and then concentrating the culture supernatant and dialyzing it against phosphate-buffered saline (PBS) with a cutoff of 6 kDa.

Serum cholesterol. Sera were incubated at 60°C for 30 min before being shipped to IDEXX Preclinical Research Services for total-cholesterol measurement.

***M. tuberculosis* infection.** A frozen stock of *M. tuberculosis* Erdman, $\sim 10^8$ CFU/ml in PBS-0.05% Tween 80, was thawed, the concentration was adjusted with PBS-0.05% Tween 80 to deliver ~ 50 CFU per mouse, and it was sonicated for 5 min in a cup horn sonifier (Branson Ultrasonics Corporation) and mice were infected in a Glass-Col Inhalation Exposure System (Glas-Col, LLC). In every experiment, two mice were sacrificed 24 h postinfection (p.i.) to confirm the delivered dose.

Bacterial load. Tissues were homogenized in PBS-0.05% Tween 80, serially diluted 10-fold over 4 to 5 log units, and plated in duplicate on Middlebrook 7H11 agar (Difco, Becton Dickinson) supplemented with oleic acid-albumin-dextrose-catalase, and cultured at 37°C, and the colonies were counted 3 weeks later.

Histopathology. Lungs were inflated and fixed with 10% buffered formalin for ≥ 24 h and then processed for staining. Tissue sections were stained with hematoxylin and eosin (H&E) or for inducible nitric oxide synthase (iNOS) production with a 1:100 dilution of anti-iNOS/NOS II rabbit polyclonal immunoglobulin G (Millipore), and bound antibody was detected with a peroxidase-based ABC staining kit according to the manufacturer's protocol (Vector Laboratories). As a negative control, sections were stained in the absence of the primary antibody. All staining was done by the Diabetes and Endocrinology Research Center histopathology core facility at the UMMS.

Fluorescence-activated cell sorter analysis. Lungs were perfused through the heart with PBS, minced, digested for 30 min at 37°C in collagenase IV (1 mg/ml) and DNase (25 μ g/ml) (both from Sigma-Aldrich), and passed through a 40- μ m cell strainer, and the remaining red blood cells were lysed with Gey's solution. Viable cells were counted using a hemocytometer and trypan blue dye staining. Lung leukocytes or splenocytes (1×10^6 to 2×10^6) were treated with Fc-blocking monoclonal antibody (clone 2.4G20; BD Bioscience Pharmingen) and then stained with allophycocyanin-cyanine 7-conjugated anti-CD3 (145-2C11), peridinin chlorophyll α -conjugated anti-CD4 (RM4-5), phycoerythrin (PE)-conjugated anti-CD8 (53.6-7), and PE-cyanine 7-conjugated anti-Gr-1 (RB6-8C5) (all from BD Bioscience Pharmingen) and allophycocyanin-conjugated anti-F4/80 (BM8; eBioscience). Flow cytometry was performed on an LSRII flow cytometer (BD Bioscience Pharmingen), 50,000 leukocyte-gated events were collected, and data analysis was done with FlowJo PC (TreeStar, Inc.). Isotype control antibodies were purchased from BD Bioscience Pharmingen and eBioscience.

Lung cytokine production. Lungs were homogenized in PBS-0.05% Tween 80; an equal volume of cell lysis buffer was added (0.5% Trion X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl₂, and 1 mM MgCl₂, pH 7.4); the lungs were vortexed, incubated for 20 min at 4°C, vortexed, and centrifuged for 10 min at $12,000 \times g$; and the supernatant was sterile filtered. Lung lysates were assayed for interleukin 1 α (IL-1 α), IL-1 β , IL-2, IL-4, IL-10, IL-17, tumor necrosis factor (TNF), macrophage inflammatory protein 1 α , and transforming growth factor beta by multiplex enzyme-linked immunosorbent assay (ELISA) (Searchlight; Pierce Biotechnology). Gamma interferon (IFN- γ) in lung lysates was measured with an ELISA kit according to the manufacturer's protocol (R&D Systems).

Ex vivo T-cell restimulation. Splenocytes were cultured for 48 h at 37°C and 5% CO₂ in medium with 4 μ g/ml concanavalin A (Sigma-Aldrich) or 2 μ g/ml *M. tuberculosis* Erdman CFP in 24-well plates with 2×10^6 cells per well in 2 ml. After 48 h, the contents of each well were sterile filtered and assayed for IFN- γ by ELISA.

In vivo antigen presentation. Leukocytes from the spleen and lymph nodes (LN) of OT-II mice were labeled with 2 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE), their viability was confirmed by trypan blue dye exclusion, CFSE labeling was confirmed by fluorescence microscopy, and ~ 15 million cells were injected intravenously into recipient mice. Twenty-four hours later, the recipient mice were injected subcutaneously (s.c.) near the left inguinal LN with 100 μ l of OVA-coated iron beads (0.1 μ g/ μ l) or with uncoated iron beads as a

negative control; 72 h later, leukocytes were isolated from the left inguinal LN. The cells were stained with peridinin chlorophyll α -conjugated anti-CD4 (clone RM4-5) and PE-conjugated anti-CD45.1 (Ly-5.1; clone A20), and 200,000 leukocyte-gated events were collected on a LSRII flow cytometer and analyzed as described above.

Statistical analysis. An F test was performed to confirm that variances between two groups were not statistically significant. Student's *t* test for samples with equal variance or unequal variance was performed as appropriate for comparisons between two groups. Comparison between more than two groups was done by analysis of variance, and a Tukey-Kramer multiple-comparison posttest was performed when analysis of variance results were significant. All tests were performed with GraphPad Instat (version 3.05 for Windows 95; GraphPad Software). *P* values of <0.05 were considered significant.

RESULTS

Hypercholesterolemia increases the TB susceptibility of *ApoE*^{-/-} mice. Mice were fed LC or HC diets in an effort to correlate TB resistance with different levels of serum cholesterol. The mean serum cholesterol for WT LC, WT HC, *ApoE*^{-/-} LC, and *ApoE*^{-/-} HC mice are shown in Fig. 1A. Serum total cholesterol of >200 mg/dl indicated hypercholesterolemia. The mice were maintained on these diets for 2 weeks before and during an experiment. Serum cholesterol measurements taken before infection and at the conclusion of experiments did not significantly differ within treatment groups ($n = 4$ or 5) (data not shown). WT and *ApoE*^{-/-} mice on LC or HC diets were infected with ~ 50 CFU of *M. tuberculosis* Erdman, and survival was monitored for 3 months (Fig. 1B). All five *ApoE*^{-/-} HC mice in this experiment died within 4 weeks after *M. tuberculosis* infection. By 3 weeks p.i., they appeared hunched, lethargic, poorly groomed, and visibly smaller than their WT LC, WT HC, and *ApoE*^{-/-} LC counterparts. On gross inspection, their lungs had large abscess-like masses with extensive inflammation and tissue necrosis visible by histology (Fig. 1C and D). All of the WT LC, WT HC, and *ApoE*^{-/-} LC mice survived until the conclusion of the experiment 3 months p.i. and showed no overt signs of illness.

WT and *ApoE*^{-/-} HC mice had comparable lung bacterial burdens up to 20 days p.i., suggesting that a defect of innate immunity or an enhanced intrinsic rate of *M. tuberculosis* replication was not responsible for the TB susceptibility of *ApoE*^{-/-} HC mice. By 30 day p.i., the bacterial lung burden reached a plateau of 6.5 log units CFU in WT HC mice while it was still increasing exponentially in *ApoE*^{-/-} HC mice, reaching 8.5 log units at the time of death (Fig. 2A). The bacterial lung burden of *ApoE*^{-/-} LC mice was ~ 0.5 log CFU greater than that of WT LC mice 30 day p.i. ($P < 0.05$; $n = 4$) (data not shown). Since control of *M. tuberculosis* growth in the lungs requires an effective adaptive immune response, these results suggested that adaptive immunity was severely compromised in *ApoE*^{-/-} HC mice.

Increased lung inflammation in *ApoE*^{-/-} mice with TB. The pattern and kinetics of lung leukocyte recruitment were investigated in *M. tuberculosis*-infected *ApoE*^{-/-} HC and WT HC mice. Lung leukocytes were isolated from the left lung lobe and the right caudal lobe by enzymatic digestion, and T-cell (CD3⁺ CD4⁺/CD8⁺), macrophage/monocyte (F4/80⁺), and granulocyte (GR-1⁺ CD3⁻ F4/80⁻) populations were measured by flow cytometry. Signs of increased lung inflammation were detectable in *ApoE*^{-/-} HC mice as early as 10 days p.i. and involved a massive influx of granulocytes (Fig. 2B and C).

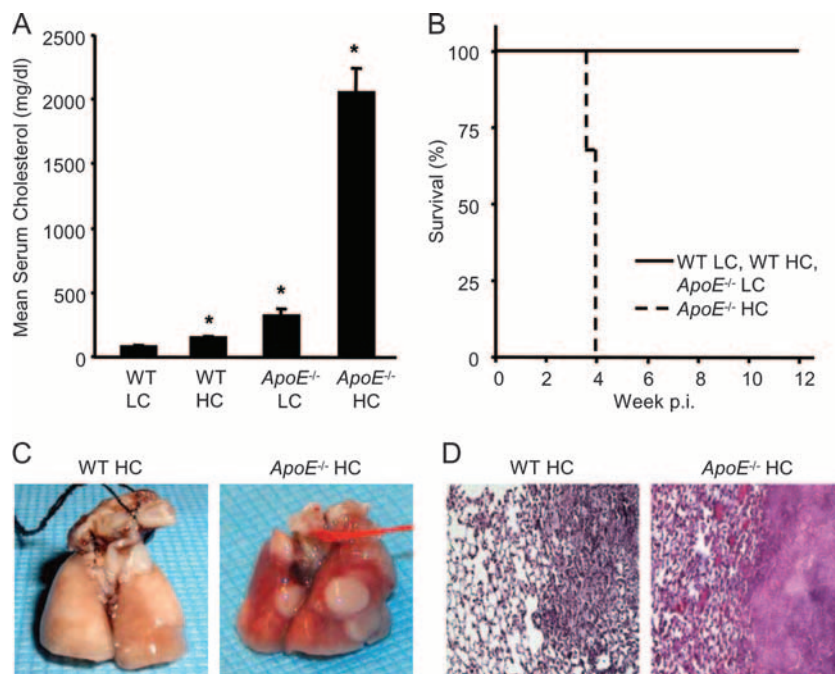


FIG. 1. *ApoE*^{-/-} HC mice are extremely susceptible to TB. (A) Serum cholesterol of WT and *ApoE*^{-/-} mice after 2 weeks on an LC or HC diet. The values are means plus standard deviations. *, $P < 0.05$ WT HC, *ApoE*^{-/-} LC, and *ApoE*^{-/-} HC mice versus WT LC mice ($n = 5$). (B) Survival times of WT and *ApoE*^{-/-} mice fed an LC or HC diet and then infected by aerosol with ~ 50 CFU *M. tuberculosis* Erdman ($n = 5$). (C) Representative lungs from WT HC and *ApoE*^{-/-} HC mice 30 days p.i. by aerosol with *M. tuberculosis*. (D) Representative lung tissue sections from WT HC and *ApoE*^{-/-} HC mice 30 days after aerosol infection with ~ 50 CFU *M. tuberculosis* stained with H&E (magnification, $\times 200$). The results shown are representative of at least two independent experiments.

Roughly 45% of the lung leukocytes of *ApoE*^{-/-} HC mice at this time point were granulocytes versus $<10\%$ for WT HC mice. Most of the granulocytes appeared to be neutrophils, with few basophils or eosinophils visible in H&E-stained lung tissue sections. Despite the massive early influx of neutrophils into the lungs of *ApoE*^{-/-} HC mice, extensive lung tissue necrosis and inflammation were not evident by histology until after 20 days p.i. While *ApoE*^{-/-} HC lungs had more T cells and macrophages than those of WT HC mice by 30 days p.i., they comprised a smaller proportion of the total lung leukocyte population than in WT HC mice. Uninfected *ApoE*^{-/-} HC mice also had a higher proportion of resident lung granulocytes (24% versus 14%) but a lower proportion of T cells (3% versus 7%) than WT HC mice ($P < 0.05$; $n = 5$). The exaggerated innate immune response and relatively low T-cell numbers despite the presence of a high antigen burden served as additional indicators that adaptive immunity was impaired in *ApoE*^{-/-} HC mice. Differences between WT LC, WT HC, and *ApoE*^{-/-} LC mice were not as dramatic as those observed with *ApoE*^{-/-} HC mice; therefore, these mice were examined 3 months p.i. The degree of lung inflammation after *M. tuberculosis* infection coincided with increasing serum cholesterol, as *ApoE*^{-/-} LC mice had a modest but statistically significant increase in lung leukocytes compared with WT LC and WT HC mice after 3 months of TB disease (Fig. 3A). There was no difference in the proportions of T-cell, macrophage/monocyte, and granulocyte populations between WT LC, WT HC, and *ApoE*^{-/-} LC mice 3 months p.i. (Fig. 3B). However, *ApoE*^{-/-} LC mice had significantly

more T cells and granulocytes than WT LC and WT HC mice (Fig. 3B).

Lung cytokine production of WT and *ApoE*^{-/-} mice with TB.

A Th1 cell-mediated immune response is required for protection against TB, and it is well established that IFN- γ is essential for resistance to TB in mice and humans (6, 10, 18). We assayed lung lysates from WT and *ApoE*^{-/-} mice on LC or HC chow for Th1, Th2, and proinflammatory cytokines. The mean IFN- γ levels in the lungs of WT LC, WT HC, and *ApoE*^{-/-} LC mice were 268 pg/ml, 253 pg/ml, and 166 mg/ml, respectively, 3 months p.i. Although *ApoE*^{-/-} LC mice had significantly lower IFN- γ production than WT LC and HC mice ($P < 0.05$; $n = 4$), it is unclear whether this small difference accounts for the fivefold-higher bacterial lung burden of *ApoE*^{-/-} LC mice than WT LC mice. Interestingly, there was no difference in lung IFN- γ production between WT HC and *ApoE*^{-/-} HC mice 20 days and 30 days p.i. (Fig. 4A). This raised the possibility that the lung macrophages of *ApoE*^{-/-} HC mice might have had a reduced capacity to respond to IFN- γ , thereby reducing their ability to kill internalized bacilli. Stimulation of macrophages to produce iNOS is one of the most important antimycobacterial functions of IFN- γ (9, 27, 12). To assess IFN- γ responsiveness, immunohistochemical staining for iNOS was performed on lung tissue sections from WT HC and *ApoE*^{-/-} HC mice 10 days, 20 days, and 30 days p.i. (Fig. 4B). Abundant iNOS production was detected in the TB lung lesions of *ApoE*^{-/-} HC mice, indicating that their macrophages retained the capacity to respond to IFN- γ .

Levels of Th1, Th2, and proinflammatory cytokines in lung

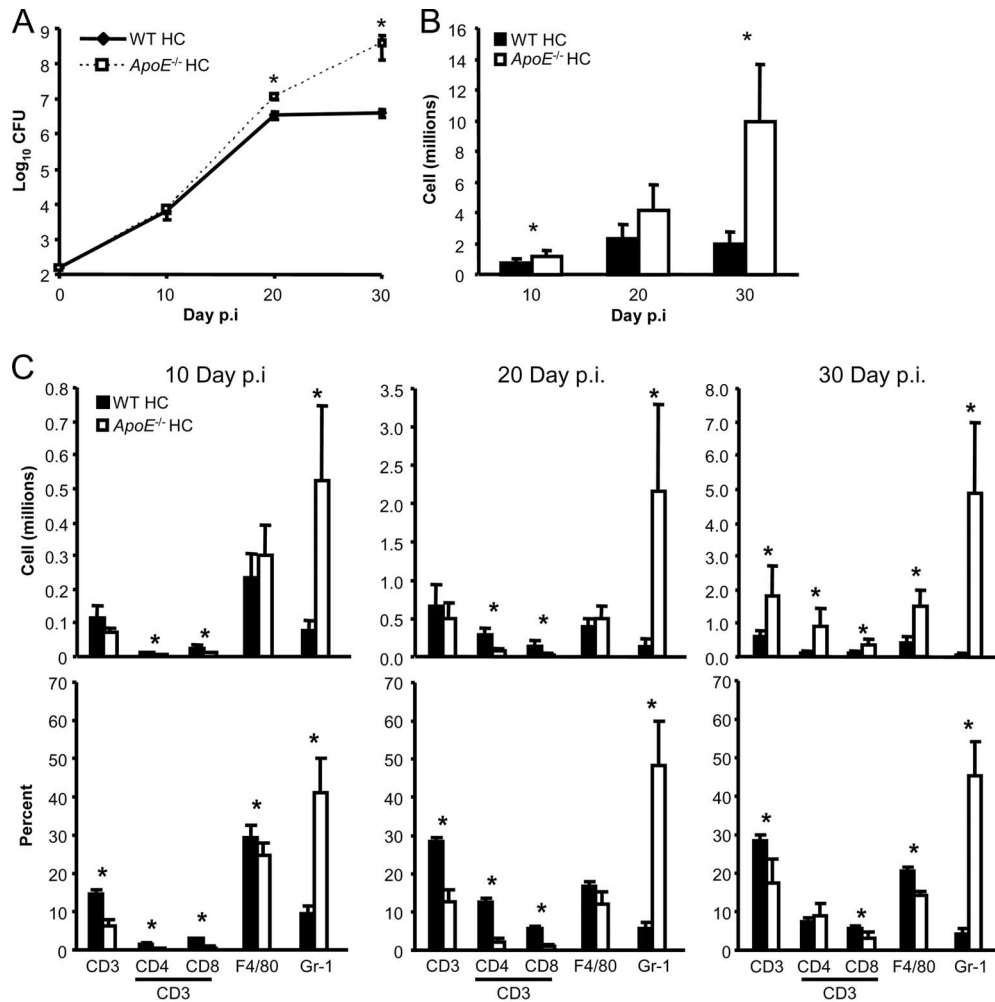


FIG. 2. Despite a massive inflammatory response, *ApoE*^{-/-} HC mice fail to control *M. tuberculosis* growth. (A) *M. tuberculosis* lung burdens of WT HC and *ApoE*^{-/-} HC mice 10 days, 20 days, and 30 days after aerosol infection. The data are presented as mean log₁₀ CFU ± standard deviations (SD). *, *P* < 0.05 (*n* = 4). (B) Lung leukocyte counts for the right caudal and left lung lobes of WT HC and *ApoE*^{-/-} HC mice 10 days, 20 days, and 30 days after aerosol infection with *M. tuberculosis*. The values are the mean cell counts expressed in millions plus SD. *, *P* < 0.05 (*n* = 5). (C) The total numbers (top graphs) and percentages (bottom graphs) of T cells (CD3⁺ CD4⁺/CD8⁺), macrophages (F4/80⁺), or granulocytes (Gr-1⁺ CD3⁻ F4/80⁻) isolated from the right caudal and left lung lobes of WT HC and *ApoE*^{-/-} HC mice 10 days, 20 days, and 30 days after aerosol infection with *M. tuberculosis* Erdman. The total number of each cell population was determined by multiplying the percentage of cells positive for the above-mentioned surface markers by the total number of lung leukocytes isolated from each mouse. The values are the mean numbers of cells expressed in millions plus SD. *, *P* < 0.05 (*n* = 5). The results shown are representative of one experiment per time point, except for the CFU data for day 30 p.i., which is representative of two independent experiments.

homogenates were similar between WT LC, WT HC, and *ApoE*^{-/-} LC mice 3 months p.i. (data not shown). Th1 and Th2 cytokine production levels were also comparable in WT HC and *ApoE*^{-/-} HC lungs, and neither group had detectable production of transforming growth factor beta 20 days p.i. Production of the inflammatory cytokines IL-1β, macrophage inflammatory protein 1α, and TNF increased dramatically in the lungs of *ApoE*^{-/-} HC mice between 20 days and 30 days p.i. (Fig. 4A). *ApoE*^{-/-} HC mice lost ~7% of their preinfection body weight, consistent with the elevated production of TNF, while WT HC mouse preinfection body weight increased ~14% by 30 days p.i. (data not shown). Interestingly, IL-17 production levels were similar in WT HC and *ApoE*^{-/-} HC mice. IL-17 is associated with neutrophilic inflammation (16) but appears not to play a significant role in the inflammation

seen in the lungs of *M. tuberculosis* Erdman-infected *ApoE*^{-/-} HC mice. The overproduction of inflammatory cytokines in *ApoE*^{-/-} HC lungs was consistent with the histology and lung leukocyte data and presumably reflected the persistent stimulation of innate immunity in the absence of an effective adaptive immune response.

Response to ex vivo antigen stimulation by WT and *ApoE*^{-/-} mice with TB. Any delay in the adaptive immune response to low-dose aerosol *M. tuberculosis* challenge in mice results in a higher bacterial lung burden before bacterial growth is restricted. To monitor the kinetics of the adaptive immune response to *M. tuberculosis* in WT HC and *ApoE*^{-/-} HC mice, splenocytes were stimulated ex vivo for 48 h with *M. tuberculosis* CFP, and IFN-γ release was measured by ELISA. Splenocytes incubated with medium or concanavalin A were

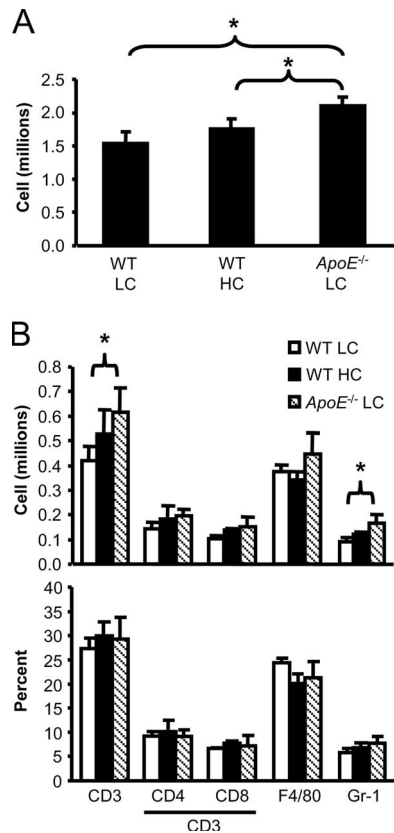


FIG. 3. Lung inflammation increases with increasing serum cholesterol. (A) Lung leukocyte counts for the right caudal and left lung lobes of WT LC, WT HC, and *ApoE*^{-/-} LC mice 12 weeks after aerosol infection with *M. tuberculosis*. The values are the mean numbers of cells expressed in millions plus standard deviations (SD). *, $P < 0.05$ ($n = 5$). (B) The total numbers (top graphs) and percentages (bottom graphs) of T cells (CD3⁺ CD4⁺/CD8⁺), macrophages (F4/80⁺), or granulocytes (Gr-1⁺ CD3⁻ F4/80⁻) isolated from the right caudal and left lung lobes of WT LC, WT HC, and *ApoE*^{-/-} LC mice 12 weeks after aerosol infection with *M. tuberculosis*. The total number of each cell population was determined by multiplying the percentage of cells positive for the above-mentioned surface markers by the total number of lung leukocytes isolated from each mouse. The values are the mean numbers of cells expressed in millions plus SD. *, $P < 0.05$ ($n = 4$). The results shown are representative of one experiment.

used as negative and positive controls, respectively. A strong IFN- γ response by T cells from WT HC mice to ex vivo CFP stimulation was seen as early as 20 days p.i., while the response by T cells from *ApoE*^{-/-} HC mice was weak at this time point (Fig. 5A). By 30 days p.i., the CFP-stimulated IFN- γ response of splenocytes from *ApoE*^{-/-} HC mice was comparable to that of WT HC mice. The spleen bacterial burdens were comparable between WT HC and *ApoE*^{-/-} HC mice 30 days p.i., 4.7 log units and 4.9 log units, respectively ($n = 4$), indicating that differences in IFN- γ responses were not caused by unequal antigen loads. The number of splenocytes in WT HC mice increased during *M. tuberculosis* infection but remained unchanged in *ApoE*^{-/-} HC mice (Fig. 5B). *ApoE*^{-/-} HC mice had more granulocytes in the spleen than WT HC mice despite having fewer total splenocytes (Fig. 5C). At 20 days and 30 days p.i., WT HC mice had almost fivefold more T cells than *ApoE*^{-/-} HC mice. Taken together, these results indicate that

by 30 days p.i., IFN- γ production on a per-T-cell basis was actually higher in the spleens of *ApoE*^{-/-} HC mice than in WT HC mice. Uninfected WT HC and *ApoE*^{-/-} HC mice had similar numbers of splenocytes, but *ApoE*^{-/-} HC mice had slightly more granulocytes (data not shown). T cells from WT LC and *ApoE*^{-/-} LC mice 3 months p.i. produced equivalent amounts of IFN- γ after ex vivo stimulation with CFP (data not shown). WT LC and *ApoE*^{-/-} LC mice had comparable numbers of splenocytes with no differences in the proportion or number of T cells, macrophages/monocytes, or granulocytes (data not shown).

In vivo antigen presentation by WT and *ApoE*^{-/-} mice. The *M. tuberculosis*-specific T-cell response of *ApoE*^{-/-} HC mice was delayed compared to that of WT HC mice, suggesting that inefficient priming of the adaptive immune response might be responsible for their increased TB susceptibility. To test this possibility, an adoptive-transfer experiment was done using leukocytes from OT-II T-cell receptor (TCR) transgenic mice. These mice express a TCR that recognizes OVA peptide (amino acids 323 to 339) in the context of a major histocompatibility complex class II I-A^b molecule (3). Their cells were distinguishable from WT and *ApoE*^{-/-} leukocytes by the allelic marker CD45.1. CFSE-labeled OT-II leukocytes were injected intravenously into WT HC and *ApoE*^{-/-} HC mice, and the next day, OVA-coated or uncoated iron beads were injected s.c. near the left inguinal LN. After 3 days, leukocytes were harvested from the left inguinal LN, and the proliferative response of OT-II T cells was measured by CFSE dilution. OT-II T cells from WT HC mice injected with OVA-coated iron beads underwent about five rounds of cell division, while significantly less proliferation of OT-II T cells was seen in identically treated *ApoE*^{-/-} HC mice (Fig. 6). No proliferation of OT-II T cells was seen in WT HC or *ApoE*^{-/-} HC mice injected with uncoated iron beads. The frequency of CD4⁺ CD45.1⁺ OT-II T cells in the inguinal LN of *ApoE*^{-/-} HC mice receiving OVA-coated iron beads was comparable to that in WT HC and *ApoE*^{-/-} HC mice that received uncoated iron beads (data not shown). This indicated equivalent seeding and survival of OT-II T cells in WT HC and *ApoE*^{-/-} HC inguinal LN. These results support the conclusion that priming of the adaptive immune response is significantly impaired in *ApoE*^{-/-} HC mice prior to *M. tuberculosis* infection.

DISCUSSION

Limited work with mouse models has demonstrated that hypercholesterolemia can have detrimental effects on the host defense. The underlying mechanisms responsible for this remain poorly understood and are likely to differ depending on the pathogen studied. The susceptibility of *ApoE*^{-/-} mice to *L. monocytogenes* and *K. pneumoniae* has been attributed to impaired innate immunity (25, 5), while defective cytotoxic-T-lymphocyte function was linked to LCMV susceptibility in *ApoE*^{-/-} and *LDL-R*^{-/-} mice (11). We investigated the impact of elevated cholesterol on host defense using an *M. tuberculosis* aerosol infection model. Unexpectedly, *ApoE*^{-/-} HC mice exhibited extreme susceptibility to *M. tuberculosis* with a survival time comparable to that of IFN- γ -deficient mice, which are the most TB-susceptible knockout mouse strain known (14, 19). Even moderately elevated cholesterol, as seen

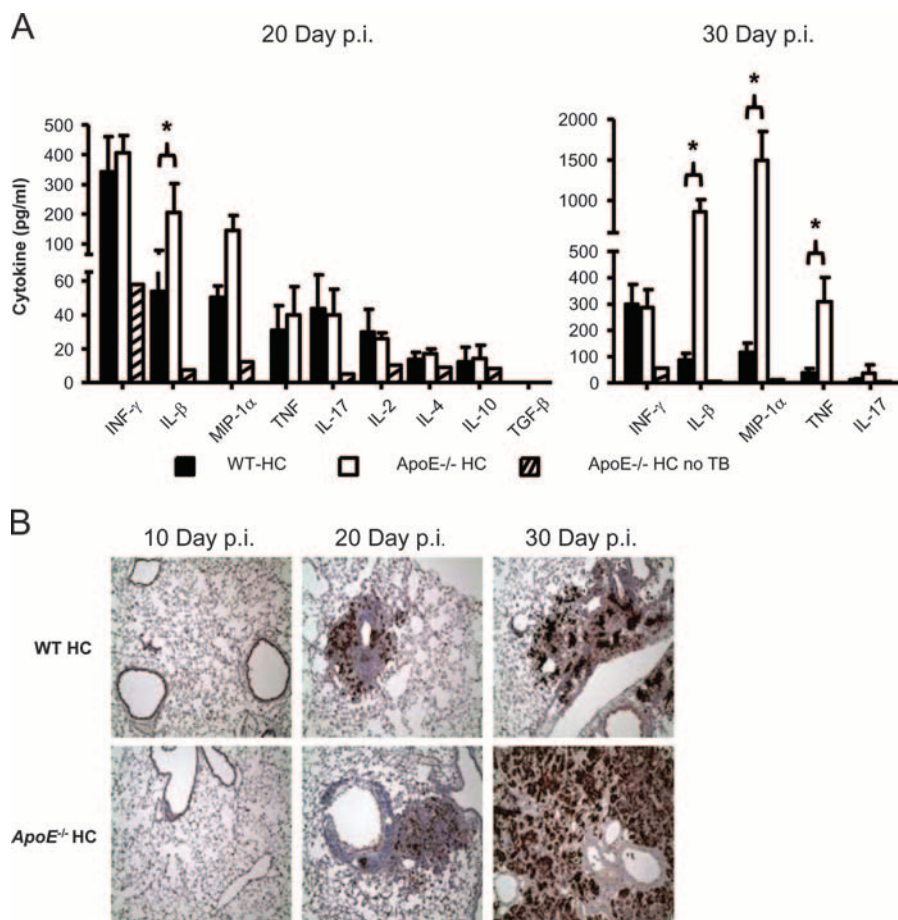


FIG. 4. Production of inflammatory cytokines in *ApoE*^{-/-} HC lungs increases with the *M. tuberculosis* burden, while iNOS production is comparable to that in WT HC lungs. (A) Cytokine production in lung homogenates from WT HC and *ApoE*^{-/-} HC mice 20 days or 30 days after aerosol infection with *M. tuberculosis*. The values are the mean cytokine concentrations expressed as pg/ml plus standard deviations. *, *P* < 0.05 (*n* = 4). Lung homogenate from an uninfected *ApoE*^{-/-} HC mouse was used as a background control. (B) Lung tissue sections from WT HC and *ApoE*^{-/-} HC mice stained for iNOS production by immunohistochemistry 10 days, 20 days, and 30 days after aerosol infection with *M. tuberculosis*. Cells expressing iNOS are identified by brown staining; magnification, $\times 40$. The lung sections shown are representative of three mice per mouse strain per time point. The results shown are representative of one experiment.

in *ApoE*^{-/-} LC mice, influenced TB immunity. The TB susceptibility of *ApoE*^{-/-} mice increased with serum cholesterol, indicating that their susceptibility was dependent on cholesterol and not ApoE deficiency per se. *ApoE*^{-/-} HC mice mount a reasonably robust antigen-specific Th1 immune response to *M. tuberculosis*, but its expression is delayed during the critical period of logarithmic bacterial growth, allowing a massive increase in the bacterial burden. Although lung inflammation was increased in hypercholesterolemic mice, dramatically so in the case of *ApoE*^{-/-} HC mice, the data suggest that the most significant impact of hypercholesterolemia on TB defense is impaired priming of the adaptive immune response.

A systemic proinflammatory state has been described in *ApoE*^{-/-} mice (4, 7). Consistent with those observations, the number of resident granulocytes and IL-1 β production were slightly elevated in the lungs of uninfected *ApoE*^{-/-} mice. The rapid influx of granulocytes into the lungs of *ApoE*^{-/-} HC mice after *M. tuberculosis* infection also suggests they are primed for inflammation. However, the role granulocytes play in controlling *M. tuberculosis* growth remains unclear, since

neutrophil depletion has been reported to cause no change in the *M. tuberculosis* lung burden in C57BL/6 mice and only a marginal increase in BALB/c mice (28, 20). Despite mounting a vigorous early inflammatory response involving heavy granulocyte infiltration of the lung, *ApoE*^{-/-} HC mice had a bacterial lung burden similar to that of WT HC mice up to 20 days after infection. Around the same time, *ApoE*^{-/-} HC mice started developing atypical massive abscess-like lesions that were heavily infiltrated by neutrophils. In contrast, established TB lesions in C57BL/6 mice are predominately composed of macrophages and T cells that are occasionally organized into granuloma-like structures. Heavy neutrophil infiltration and necrosis have also been described in TCR- α/β ^{-/-} mouse TB lung lesions (14); however, unlike *ApoE*^{-/-} HC mice, iNOS expression was greatly reduced in these mice. The inability of *ApoE*^{-/-} HC mice to control *M. tuberculosis* growth may have exacerbated their inflammatory response to *M. tuberculosis*, leading to the massive lung tissue destruction observed by 30 days p.i.

Following aerosol infection of WT mice, *M. tuberculosis*

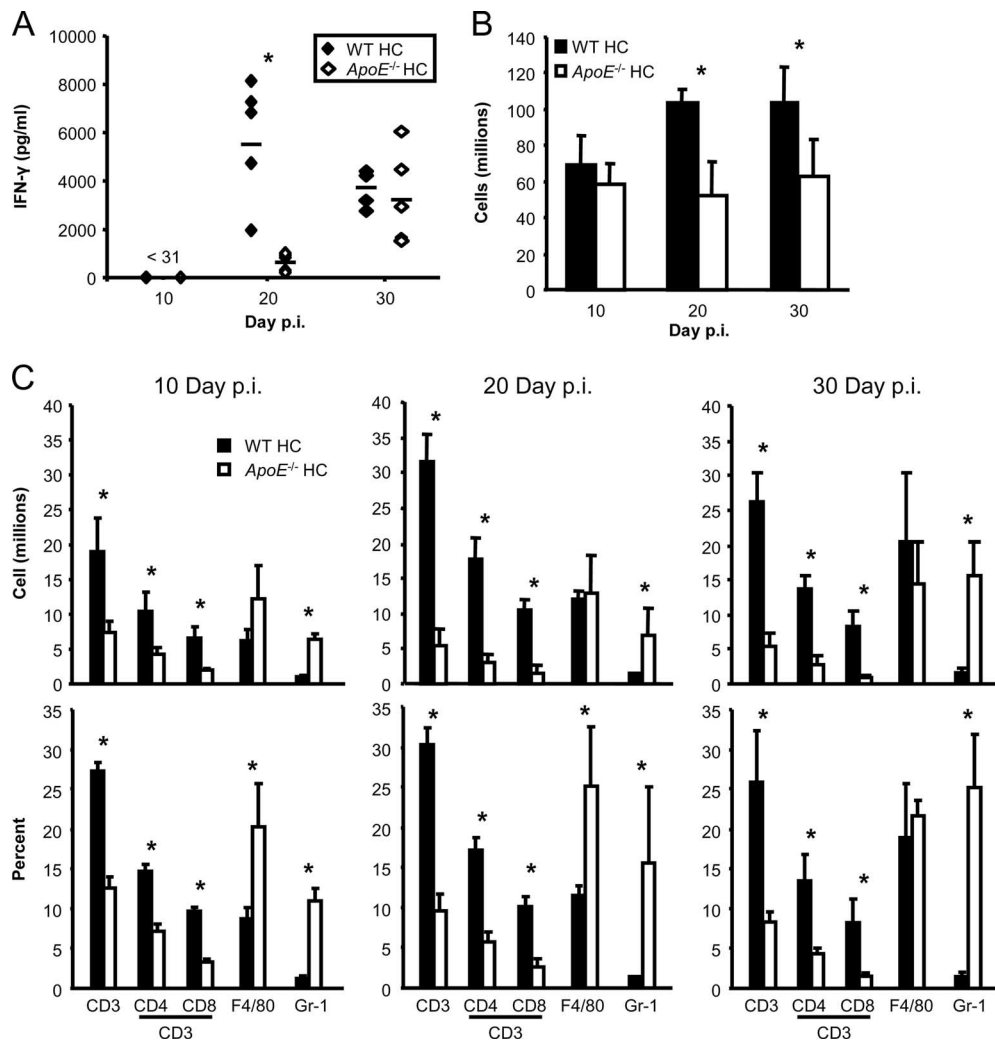


FIG. 5. Delayed *M. tuberculosis* antigen-specific T-cell response in *ApoE*^{-/-} HC mice. (A) Amounts of IFN- γ released by splenocytes 10 days, 20 days, and 30 days after aerosol infection with *M. tuberculosis* from WT HC and *ApoE*^{-/-} HC mice that were cultured for 48 h with 2 μ g/ml *M. tuberculosis* Erdman CFP. Individual measurements are shown, and the mean concentration is indicated with a solid line. Day 10 was below the lower limit of detection of 31 pg/ml. *, $P < 0.05$ ($n = 5$). (B) Splenocyte counts from WT HC and *ApoE*^{-/-} HC mice 10 days, 20 days, and 30 days after aerosol infection with *M. tuberculosis*. The values are the mean splenocyte counts plus standard deviations (SD). *, $P < 0.05$ ($n = 5$). (C) The total numbers (top graphs) and percentages (bottom graphs) of T cells (CD3⁺ CD4⁺/CD8⁺), macrophages (F4/80⁺), or granulocytes (Gr-1⁺ CD3⁻ F4/80⁻) isolated from the spleens of WT HC and *ApoE*^{-/-} HC mice 10 days, 20 days, and 30 days after aerosol infection with *M. tuberculosis*. The total number of each cell population was determined by multiplying the percentage of cells positive for the above-mentioned surface markers by the total number of splenocytes isolated from each mouse. The values are the mean numbers of cells expressed in millions plus SD. *, $P < 0.05$ ($n = 4$). The results shown for panels A and B are representative of two experiments, and the results for panel C are representative of one experiment.

grows exponentially in the lungs for about 20 days, after which the bacterial load is held at a plateau by adaptive immunity. Control of TB requires an effective Th1 adaptive immune response, and particularly IFN- γ , to enhance the antimycobacterial functions of macrophages. Mice with impaired adaptive immunity, e.g., SCID mice, OVA-specific TCR transgenic mice, or IFN- γ -deficient mice, succumb rapidly to TB (6, 14, 17, 19). C57BL/6, the background strain of the *ApoE*^{-/-} mice used in our study, is a TB-resistant mouse strain with an expected survival time of >200 days after low-dose aerosol infection (14). Adaptive immunity to TB was severely impaired in *ApoE*^{-/-} HC mice, since they were unable to restrict *M. tuberculosis* growth and died \sim 28 days p.i. Hypercholesterol-

emia did not prevent an antigen-specific T-cell response to *M. tuberculosis* from developing, but its expression was significantly delayed in *ApoE*^{-/-} HC mice. Comparable amounts of Th1- and Th2-related cytokines were found in the lungs of WT and *ApoE*^{-/-} mice with TB, indicating that hypercholesterolemia did not predispose the mice to a less protective Th2 response. The ability of macrophages to respond to appropriate activation by IFN- γ did not appear to be compromised by hypercholesterolemia, since abundant iNOS production was detected in *ApoE*^{-/-} mice during the course of *M. tuberculosis* infection.

Severe hypercholesterolemia impaired priming of the adaptive immune response, since OT-II T cells adoptively trans-

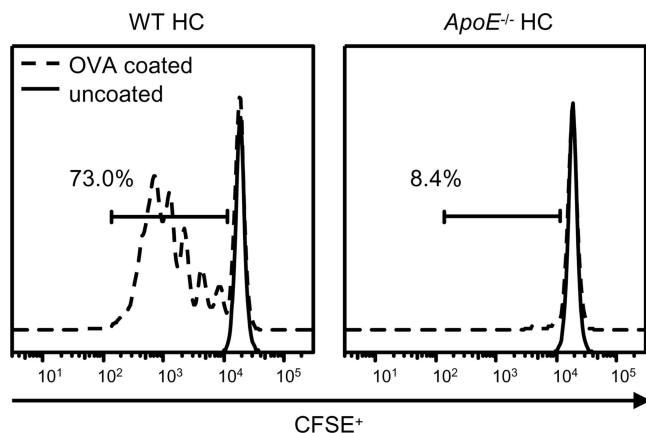


FIG. 6. In vivo antigen presentation is impaired in *ApoE*^{-/-} HC mice. Shown are representative histograms of CFSE fluorescence of OT-II CD45.1⁺ CD4⁺ cells recovered from the left inguinal LN of WT HC and *ApoE*^{-/-} HC mice. OT-II cells were stimulated in vivo for 3 days by s.c. injection of OVA-coated or uncoated iron beads near the left inguinal LN. The results are representative of three mice per group that were stimulated with OVA-coated beads; 66.6% and 13.4% of OT-II CD45.1⁺ CD4⁺ cells underwent ≥ 1 cell division in WT HC and *ApoE*^{-/-} HC mice, respectively. *, $P < 0.05$ ($n = 3$). The results shown are representative of one experiment.

ferred into uninfected *ApoE*^{-/-} HC mice proliferated weakly after stimulation with OVA. Reduced proliferation of LCMV-specific transgenic CD4⁺ T cells to their cognate peptide was also recently reported in *ApoE*^{-/-} mice fed a high-fat/cholesterol diet (29). DCs play a critical role in the priming of the adaptive immune response, and their impairment by high cholesterol would be consistent with the delayed immune response to *M. tuberculosis* and subsequent uncontrolled bacterial growth observed in *ApoE*^{-/-} HC mice. This idea is also supported by a recent report (31) that transient in vivo depletion of CD11c⁺ DCs in mice delays their antigen-specific immune response to *M. tuberculosis* and increases their bacterial burden, although not as severely as seen in our experiments. Angeli et al. (2) reported that oxidized LDL (ox-LDL) interferes with the normal trafficking of DCs from the skin to draining LN in *ApoE*^{-/-} mice fed a high-fat diet. Inefficient migration of lung DCs to the draining LN of *ApoE*^{-/-} HC mice after *M. tuberculosis* infection could also explain their delayed adaptive immune response to TB. Recently, ox-LDL was reported to increase the susceptibility of *ApoE*^{-/-} mice fed a high-fat/cholesterol diet to *Leishmania major* by priming CD8 α ⁻ myeloid DCs to induce a nonprotective Th2 immune response (29). However, *ApoE*^{-/-} HC mice with TB mounted a robust, albeit delayed, Th1 immune response, and IL-4 production in their lungs was not elevated compared to WT HC mice. Although ox-LDL may prime CD8 α ⁻ myeloid DCs to induce a Th2 immune response, it does not appear to be responsible for the increased TB susceptibility of *ApoE*^{-/-} HC mice. Our data indicate that hypercholesterolemia negatively impacts the TB host defense by interfering with the priming of the adaptive response, which could reflect impairment of DC antigen uptake or processing, migration to LN, or antigen presentation and costimulatory ligand expression. The molecular basis for this effect of elevated cholesterol remains to be discovered. A number of biochemical pathways for cholesterol-mediated pa-

thology at the cellular level have been proposed (reviewed in reference 30) and will direct future investigation in our model. While the strong antigen-specific T-cell response in the spleen and heavy recruitment of T cells to the lungs 30 days p.i. argues against gross impairment of T cells in *ApoE*^{-/-} HC mice, the impaired priming of the adaptive immune response could be masking more subtle T-cell deficits. Future studies will examine this possibility in greater detail.

The hypercholesterolemic environment in *ApoE*^{-/-} mice might directly support more robust *M. tuberculosis* growth by increasing nutrient availability. Van der Geize et al. (32) recently reported that *M. tuberculosis* has the capacity to take up and use cholesterol as a source of energy. Further, many of the *M. tuberculosis* genes related to cholesterol uptake and catabolism have been reported to be upregulated 2 to 4 weeks p.i. (26). While *ApoE*^{-/-} HC mice had a greater bacterial lung burden than WT HC mice beginning 20 days p.i., their delayed *M. tuberculosis* antigen-specific immune response indicates that TB susceptibility is not likely to be exclusively due to a putative nutrient affect. Whether hypercholesterolemia promoted rapid *M. tuberculosis* growth and compounded the consequences of the delayed Th1 immune response by *ApoE*^{-/-} HC mice is unknown. Further investigation will be required to determine what impact, if any, hypercholesterolemia has on the growth rate of *M. tuberculosis* in vivo.

The increased TB susceptibility of hypercholesterolemic mice suggests that there may be heretofore-unappreciated health risks associated with elevated cholesterol in people. An especially vulnerable group may be people with diabetes mellitus. Diabetes is known to increase TB susceptibility (13, 21), and roughly half of the diabetics in the United States have high cholesterol (8). However, Perez-Guzman et al. (22) reported that TB patients receiving a high-cholesterol diet responded to antimycobacterial therapy with a higher sputum sterilization rate than patients on a normal diet. Patients on the high-cholesterol diet had borderline high cholesterol, ~ 210 mg/dl. In our study *ApoE* knockout mice had substantially higher serum total cholesterol whether they were on an LC or an HC diet; cholesterol was elevated prior to infection, and they did not receive antimycobacterial therapy. Further, our data indicate that hyperlipidemia has its greatest impact on the initiation of the adaptive immune response; whether cholesterol influences preexisting adaptive immune responses is unknown. Based upon our observations and the reports by Van der Geize et al. (32) and Sasseti et al. (26) that *M. tuberculosis* has the ability to use cholesterol as an energy source, we suggest exercising caution when considering cholesterol treatment of TB patients.

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