ABCA1 promotes the efflux of bacterial LPS from macrophages and accelerates recovery from LPS-induced tolerance[S]

Patricia A. Thompson,* Karine C. Gauthier,[1,†,§] Alan W. Varley,* and Richard L. Kitchens[2,*]

Department of Internal Medicine,* Department of Pharmacology,[†] and Howard Hughes Medical Institute,[§] University of Texas Southwestern Medical Center, Dallas, TX

Abstract **Macrophages play important roles in both lipid metabolism and innate immunity. We show here that macrophage ATP-binding cassette transporter A1 (ABCA1), a transporter known for its ability to promote apolipoprotein-dependent cholesterol efflux, also participates in the removal of an immunostimulatory bacterial lipid, lipopolysaccharide (LPS). Whereas monocytes require an exogenous lipoprotein acceptor to remove cell-associated LPS, macrophages released LPS in the absence of an exogenous acceptor by a mechanism that was driven, in part, by endogenous apolipoprotein E (apoE). Agents that increased ABCA1 expression increased LPS efflux from wild-type but not ABCA1-deficient macrophages. Preexposure of peritoneal macrophages to LPS for 24 h increased the expression of ABCA1 and increased LPS efflux with a requirement for exogenous apolipoproteins due to suppression of endogenous apoE production. In contrast, LPS preconditioning of ABCA1-deficient macrophages significantly decreased LPS efflux and led to prolonged retention of cell-surface LPS. Although the initial response to LPS was similar in wild-type and ABCA1-deficient macrophages, LPS-induced tolerance was greater and more prolonged in macrophages that lacked ABCA1. Our results define a new role for macrophage ABCA1 in removing cell-associated LPS and restoring normal macrophage responsiveness.**—Thompson, P. A., K. C. Gauthier, A. W. Varley, and R. L. Kitchens. **ABCA1 promotes the efflux of bacterial LPS from macrophages and accelerates recovery from LPS-induced tolerance.** ***J. Lipid Res.*** **2010.** 51: **2672–2685.**

Supplementary key words ATP-binding cassette transporter A1 • apolipoprotein • cytokine • endotoxin • gene reprogramming • immunosuppression • inflammation • lipopolysaccharide • signaling

ATP-binding cassette transporter (ABC)A1 mediates the efflux of cellular cholesterol and phospholipids in association with lipid-poor apolipoproteins, principally apolipoprotein (apo)A-I, by generating nascent HDL particles (reviewed in Refs. 1 and 2). ABCA1 is a major determinant of plasma HDL levels, and its activity in the liver and small intestine is responsible for the generation of most circulating HDL. Loss of function mutations in humans (e.g., Tangier disease) and mice are associated with HDL deficiency. In addition to maintaining HDL levels, the cardioprotective effects of ABCA1 have also been attributed to its ability to promote cholesterol efflux from macrophages in the artery wall, thereby helping to limit the accumulation of excess cellular cholesterol, which can promote inflammation and toxicity. Recent reports suggest that the cholesterol transporters ABCA1 and ABCG1 may also decrease the macrophage inflammatory response to lipopolysaccharides (LPS) (3–7) and that ABCG1 may decrease macrophage sensitivity to other microbial stimuli recognized by various Toll-like receptors (5). These studies showed that transporter-deficient macrophages had significantly higher cytokine and chemokine responses to LPS compared with control macrophages, and they showed

This work was supported by National Institutes of Health Grant AI-45896 (National Institute of Allergy and Infectious Diseases). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

Manuscript received 7 April 2010 and in revised form 15 May 2010.

Published, JLR Papers in Press, May 15, 2010
DOI 10.1194/jlr.M007435

Abbreviations: 18S rRNA, 18S ribosomal RNA; ABC, ATP-binding cassette transporter; AOAH, acyloxyacylhydrolase; apo, apolipoprotein; B6, C57Bl/6; CCL, chemokine (C-C motif) ligand; COX-2, cyclooxygenase-2; *E. coli*, *Escherichia coli*; Fpr1, formyl peptide receptor 1; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; KO, knockout; LBP, LPS-binding protein; LPS, lipopolysaccharide; LXR, liver X receptor; mCD14, membrane CD14; MCP-1, monocyte chemotactic protein-1; M-CSF, macrophage colony-stimulating factor; PBMC, peripheral blood mononuclear cell; PMA, phorbol myristate acetate; Ptgs2, prostaglandin-endoperoxide synthase 2; RXR, retinoid X receptor; sCD14, soluble CD14; SFM, serum-free medium; siRNA, small inhibitory RNA; TGFβ, transforming growth factor beta; Tlr4, Toll-like receptor 4; TNF, tumor necrosis factor; VD_3, 1,25-dihydroxyvitamin D_3; WT, wild-type.

[1] Present address of K. C. Gauthier: Institut de Génomique Fonctionnelle de Lyon, Université de Lyon, Université Lyon 1, CNRS, INRA, Ecole Normale Supérieure de Lyon, 46, allée d'Italie, 69364 Lyon Cedex 07, France.

[2] To whom correspondence correspondence should be addressed.
e-mail: rlkitchens.ut@gmail.com

[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three figures and three tables.

evidence that transporter-dependent downregulation of inflammatory responses is mechanistically linked to sterol efflux.

The control of ABCA1 expression is complex and highly regulated by multiple transcriptional and posttranscriptional processes, including those that influence protein activity and stability (1). Transcriptional activation is induced by oxysterols via the activation of liver X receptors LXRα and LXRβ, which form heterodimers with retinoid X receptors (RXR) and bind to response elements in the ABCA1 gene promoter (8). LXR activation also induces ABCG1 and apoE (9). ABCA1 mRNA and protein levels are also increased by cAMP (10), interleukin (IL)-10 (11), transforming growth factor (TGF)β (12), and tumor necrosis factor (TNF) (13). However, reports on the effects of LPS are conflicting. In one study, LPS increased ABCA1 expression by an LXR-independent pathway in the human THP-1 premonocyte cell line and in mouse liver in vivo, whereas ABCG1 expression was decreased (14). In other studies, LPS decreased macrophage ABCA1 expression (15–18).

LPS (also called endotoxin) is among the most potent of the immunostimulatory bacterial lipids. Host recognition of LPS by Toll-like receptor 4 (Tlr4) induces a large array of genes whose products have inflammatory, anti-inflammatory, and antimicrobial activities. Sensitive host responses to LPS are orchestrated by several proteins. LPS-binding protein (LBP) (19), a secreted protein found in the plasma and extravascular fluids, transfers LPS to CD14 (20, 21), an LPS binding receptor that exists in soluble or membrane-bound forms. CD14 greatly increases host cell sensitivity to LPS by transferring LPS to the MD-2/Tlr4 receptor complex (22, 23). Sentinel responses to LPS often confer resistance to infection, whereas the response to LPS during uncontrolled infection contributes to the pathophysiology of severe sepsis and septic shock. Therefore, animal hosts have evolved mechanisms for controlling LPS responses. Endotoxin tolerance or gene reprogramming (reviewed in Refs. 24 and 25) is thought to be an important mechanism for minimizing inflammation-induced damage during recovery from bacterial infection. During the tolerant or gene reprogrammed state, the vast majority of inflammation-inducing genes are suppressed, whereas other subsets of LPS-responsive genes (such as those involved in antimicrobial effectors mechanisms) remain LPS-inducible due to chromatin modifications (26). The tolerant state has been associated with a high risk of secondary infection and mortality, and recovery from tolerance has been associated with survival. Endotoxin tolerance has also been identified in noninfectious settings such as smoking, trauma, surgery, pancreatitis, alcoholic cirrhosis, and cancer, some of which may be associated with gut translocation of endotoxin.

One important mechanism for recovery from endotoxin tolerance involves the ability of macrophages and other phagocytes to rapidly internalize LPS and partially degrade it over a period of days. This critical catabolic step is performed by acyloxyacylhydrolase (AOAH), a phagocyte enzyme that inactivates LPS by removing secondary fatty acyl chains from its lipid A moiety (27). Studies with AOAH-deficient mice have revealed that LPS can persist in cells and tissues, and if not inactivated, the resulting chronic LPS stimulation produces prolonged macrophage tolerance to LPS and other microbial stimuli accompanied by harmful immunosuppression (28) and chronic pathologies involving other cells and tissues (29, 30). The regulatory mechanisms that maintain the tolerant state are exceedingly complex (reviewed in Ref. 24) and include several negative feedback loops that inhibit the Tlr4 signaling pathway at multiple levels. Other regulatory mechanisms, such as those involved in chromatin modifications, have positive or negative effects that are gene-specific. The recovery from tolerance presumably occurs when the macrophage has disposed of enough of its bioactive LPS to turn off Tlr4 signaling (28).

Our previous studies have revealed another mechanism that removes cellular LPS. We found that HDL and other plasma lipoproteins promote the release of LPS from the monocyte cell surface (31), a process that requires an LPS-binding acceptor (e.g., HDL) and is accelerated by an LPS transfer protein, principally soluble CD14 (sCD14) (31, 32). LPS efflux from monocytes is accompanied by decreased cytokine responses to LPS that has already bound to the cell; the abilities of sCD14 (32) and LBP (33) to attenuate cell responses can occur by different mechanisms that both move LPS away from MD-2/Tlr4.

In the present study, we show that LPS efflux from macrophages occurs by a different mechanism that does not require exogenous LPS acceptors or transfer proteins. We hypothesized that endogenous (macrophage-produced) apoE might play a role in LPS efflux, because it (a) is one of the most highly expressed macrophage genes (34, 35), (b) plays a role in cholesterol and phospholipid efflux (36–38), and (c) can bind LPS (39). We also hypothesized that ABCA1 is involved, because previous studies have shown that ABCA1 is a receptor for apoE and other exchangeable apolipoproteins (40, 41) and that ABCA1 promotes apolipoprotein-mediated cholesterol and phospholipid efflux (1, 2). Our results reveal the complexity of macrophage LPS efflux, and they define new roles for ABCA1 and apolipoproteins in removing cell-associated LPS and accelerating macrophage recovery from LPS-induced tolerance.

MATERIALS AND METHODS

Experimental subjects and cells

Wild-type (WT) C57BL/6J mice (stock #002207), mice with targeted deletions of the ABCA1 gene (ABCA1 KO or ABCA1$^{-/-}$) (B6:DBA/1 background; stock #003897) and the apoE gene (apoE KO or apoE$^{-/-}$) (C57Bl/6J background; stock # 002052) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice with targeted deletions of the LXRα and LXRβ genes (C57BL/6:129Sv background) were generated in the Mangelsdorf lab as previously described (42). ABCA1$^{-/-}$ mice derived from a different source (43) were also utilized after being backcrossed onto the C57Bl/6:129/Sv background as described (44). Peritoneal macrophages were isolated by lavage 4 days after intraperitoneal injection of 1.5 ml of sterile Brewer Thioglycollate

Medium (Difco). The peritoneal cells were counted using a hemocytometer and were cultured in 24-well plates (4×10^5 cells/well) in 0.5 ml of RPMI 1640 (Cellgro; Fisher Scientific) containing 10% heat-inactivated FBS (Hyclone, defined; Fisher Scientific) with penicillin and streptomycin (complete medium) (45) for 3–4 h to obtain adherent macrophages. The animal protocol was approved by the Institutional Animal Care and Use Committee (University of Texas Southwestern Medical Center). Human peripheral blood was obtained by informed consent from healthy volunteers according to protocols approved by the Institutional Review Board. HDL ($1.063 < d < 1.21$ g/cc) was prepared from freshly drawn blood of three healthy volunteers by ultracentrifugal flotation (31). Normal human monocytes were prepared from peripheral blood mononuclear cells (PBMC) isolated on Histopaque 1077 (Sigma) by adherence to plastic for 1–2 h. Human macrophages were cultured either in suspension or attached to culture plates in complete medium containing 50 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF) (Sigma) for 5–7 days. For cultures in suspension, the adherent monocytes were lifted from 10 cm culture plates by incubating the cells briefly with PBS containing 1 mM EDTA and were then cultured in Teflon beakers as described above. THP-1 cells (a human premonocyte cell line) were cultured in 0.05 μM 1,25 dihydroxyvitamin D_3 (VD_3) (BioMol, Plymouth Meeting, PA) for 3 days to induce mature monocyte characteristics (33). THP-1 cells were differentiated into adherent macrophages by adding 100 nM phorbol myristate acetate (PMA) to the above culture for the first 24 h followed by 48 h in VD_3 alone. After culture in PMA, the cells spread and adhered tightly to the plate, and they stopped proliferating.

LPS and reagents

LPS was provided by Robert Munford (University of Texas Southwestern Medical Center). The unlabeled LPS was from *Escherichia coli* (*E. coli*) O14 (Ra structure) (28). Biosynthetically labeled *E. coli* LCD25 [^{3}H]LPS (1.5×10^6 dpm/μg) (Ra structure) was labeled in the fatty acyl chains (46). Biosynthetically labeled *Salmonella typhimurium* PR122 (smooth structure) [^{3}H/^{14}C]LPS (8.5×10^5 dpm ^{3}H/μg LPS in galactose residues of the polysaccharide chain and 0.38×10^5 dpm ^{14}C/μg in the lipid A glucosamine backbone) (smooth LPS structure) contained no radioactivity in the fatty acyl chains. Biosynthetically labeled *Salmonella typhimurium* PR122 (Rc structure) [^{3}H/^{14}C]LPS (1.2×10^5 dpm ^{3}H/μg LPS in the fatty acyl chains and 0.082×10^5 dpm ^{14}C/μg LPS in the lipid A glucosamine backbone) was used to measure LPS deacylation in macrophages using the ^{3}H/^{14}C ratio in the ethanol-insoluble fraction as previously described ("Method 2" in Ref. 30). Other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

LPS efflux assays

[^{3}H]LPS was bound to adherent peritoneal macrophages in 24-well plates by incubating them with 100 ng/ml [^{3}H]LPS for 10 min at 37°C. The cells were then placed on ice, washed, and incubated in serum-free medium (SFM) containing RPMI 1640, 20 mM HEPES buffer (pH 7.4), and 0.1 mg/ml BSA for a defined amount of time at 37°C in a CO_2 incubator. The cells were then placed on ice, and radioactivity was measured in the culture supernatants and cells by liquid scintillation counting. Typically, the macrophages took up 1500–2000 dpm of LPS/well when LCD25 [^{3}H]LPS was used. In experiments using T0901317 and/or cAMP, the cells were preincubated with these compounds in complete medium (or medium containing 5% lipoprotein-deficient FBS) for 16–24 h, and the drugs were also added to the appropriate SFM-containing cultures during the LPS efflux assay. In experiments that involved LPS conditioning, the cells were preincubated with unlabeled O14 LPS for 24 h and washed before adding the [^{3}H]LPS. To distinguish between cell-surface bound and internalized [^{3}H]LPS, the cells were incubated on ice with 0.02% proteinase K in PBS for 30 min to release LPS that was bound to surface proteins as previously described (47). This treatment did not cause detachment of the macrophages from the culture dish and did not cause membrane permeability to trypan blue. Radioactivity was measured in the proteinase K supernatants and in the cells after detaching the cells in PBS with a cell scraper. In experiments in which the efflux time course exceeded 1 h, the effluxed and cell-associated [^{3}H]LPS or [^{3}H/^{14}C]LPS was precipitated with ethanol as previously described (30) to measure LPS, which is ethanol-insoluble, and to exclude ethanol-soluble degradation products such as free fatty acids.

LPS efflux from human monocytes or macrophages in suspension was performed as previously described (31, 32). The cells were allowed to take up [^{3}H]LPS (100 ng/ml) for 10 min at 37°C; they were then washed and incubated in SFM for varying amounts of time. The release of [^{3}H]LPS into the culture supernatant is expressed as the percentage of the total initial cell-associated [^{3}H]LPS. LPS efflux from THP-1 macrophages was performed using adherent cells as described above for peritoneal macrophages.

ApoE inhibition

To inhibit apoE expression by small inhibitory RNA (siRNA) knockdown, human macrophages were taken from Teflon beakers on day 2 of the above protocol, and 3×10^6 cells/tube were electroporated with 2 μM apoE siRNA (sense 5′-GGAGUUGAAGGCCUACAAAtt-3′; antisense 5′-UUUGUAGGCCUUCAACUCCtt-3′; ID #41694, Ambion, Inc., Austin, TX) or control siRNA (Negative control #1 siRNA, Ambion #4611) using the Amaxa Human Monocyte Nucleofector Kit and electroporator according to the manufacturer's instructions (Lonza, Basel, Switzerland). The mRNA analysis and measurement of apoE secretion were performed after an additional three days of culture in complete medium. The amount of apoE produced by the control macrophages in 48 h varied among the three donors (150, 410, and 607 ng/ml/10^6 electroporated cells), whereas apoC-I production was only 2–7% that of apoE and was not inhibited by the siRNA (data not shown).

In other experiments, antibodies to apoE were used to inhibit endogenous apoE activity. [^{3}H]LPS was bound to cultured human macrophages as described above. The cells were washed with cold SFM and incubated for 30 min at 4°C in SFM containing control or anti-apoE antibodies [20 μg/ml each of mouse monoclonal antibody (H11004M) and rabbit polyclonal (K741180B) antibody (Meridian Life Science/BioDesign, Saco, ME); controls contained nonimmune control mouse IgG1 and rabbit IgG (Sigma)]. The mixtures were then warmed to 37°C for 15 min to allow the release of [^{3}H]LPS.

LPS response and recovery from tolerance

Peritoneal macrophages were cultured for 16 h in 24-well plates (4×10^5 cells/well) as described above. The adherent cells were incubated with 10 ng/ml of unlabeled *E. coli* O14 LPS or control buffer for 24 h in complete medium. The cells were then washed twice, incubated with fresh complete medium for 0–5 days, and challenged with 10 ng/ml LPS for 2 h in experiments to measure mRNA or for 6 h in experiments to measure cytokine protein production. The plates were placed on ice, the supernatants were removed, and the cells were lysed with RLT buffer (RNeasy kit, Qiagen, Valencia, CA) to isolate total RNA. In experiments that measured cytokine protein production, the cells were lysed in PBS containing 1% Triton X-100, and the cell

protein was measured by the BCA (bicinchoninic acid) method (Pierce Chemical Co.) using BSA as a standard.

mRNA analysis

Total RNA (RNeasy kit) was reverse transcribed by Superscript II (Invitrogen, Carlsbad, CA) using random hexamer primers. In some experiments, total RNA was reverse transcribed using the iScript cDNA Synthesis Kit containing oligo(dT) and random hexamer primers (BioRad, Hercules, CA). Relative levels of mRNA were quantitated using Sybr Green Master Mix in a Model 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) as previously described (45). Analysis of decreasing amounts of total RNA confirmed that real-time PCR measurements were within the dynamic range for each gene. The data were analyzed by the ddCt method after validation of each primer pair by the standard curve method; the results were normalized by levels of 18S rRNA. PCR primers used are shown in supplementary Table I. Melting point analyses performed at the end of each PCR run revealed a single amplification product for each primer pair. The fold change in mRNA level was measured relative to the unstimulated WT control for each gene. The baseline mRNA levels were undetectable for some genes; in these cases, we arbitrarily assigned a threshold cycle number of 40.

Western blotting and ELISA

Total membrane fractions of peritoneal macrophages were prepared as previously described (45), and 10 μg of membrane protein in SDS-PAGE sample buffer was warmed to 95°C for 5 min for apoE samples and 25°C for ABCA1 samples. The samples were fractionated on SDS-PAGE gels and transferred to Immobilon-P membranes as described (45). The membranes were probed with affinity purified rabbit anti-ABCA1 antibodies (#NB400-105, Novus Biologicals, Littleton, CO) or anti-apoE antibodies (45) and detected by enhanced chemiluminescence (45). Secreted human apoE and apoC-I were measured by ELISA as described (45). TNF, IL-6, and RANTES [chemokine (C-C motif) ligand 5 (Ccl5)] were measured in culture supernatants by ELISA. The RANTES ELISA set was from R and D Systems (Minneapolis, MN), and the other sets were from BD Biosciences (San Jose, CA).

Statistics

The data were analyzed using Prism 5.0 software from GraphPad (San Diego, CA) with error bars denoting standard deviation (SD), standard error of the mean (SEM), or 95% confidence interval (CI). Significant differences from controls were determined as two-tailed *P* values of the unpaired *t*-test.

RESULTS

ABCA1 and endogenous apoE promote LPS efflux from macrophages

We previously showed that the rapid release of cell surface-bound LPS from human monocytes requires an LPS transfer protein and a lipoprotein acceptor (e.g., soluble CD14 and HDL) (31). Here we show that the differentiation of monocytes into macrophages alters the mechanism of LPS efflux. **Fig. 1A** shows that after normal human monocytes had differentiated into macrophages by culturing them for 5–7 days, they released cell-associated LPS much more rapidly in the absence of plasma components and without an exogenous acceptor. LPS efflux under these conditions was also significantly elevated in mouse peritoneal macrophages and human THP-1 macrophages compared with that of THP-1 monocytes and normal human monocytes.

To investigate the role of apoE in LPS efflux from macrophages, we first used real-time PCR to quantitate apoE mRNA expression. As shown in **Table 1**, we found that cultured human macrophages expressed the highest levels of apoE among the human cells tested, and as expected, monocytes expressed low levels of apoE. To test the hypothesis that endogenous apoE might play a role in LPS efflux, we attempted to inhibit its activity with anti-apoE antibodies and its expression using siRNA. As shown in Fig. 1B, preincubation of cultured human macrophages with antibodies to apoE significantly decreased [^{3}H]LPS efflux from the cells in serum-free medium that did not contain any exogenous apoE. ApoE siRNA knockdown experiments yielded similar results. As shown in Fig. 1C, incubation of macrophages with apoE siRNA decreased apoE protein production by 95%; however, this decreased LPS efflux by only 32%. The data indicate that although endogenous apoE plays a significant role in LPS efflux, the majority of LPS efflux from cultured human macrophages is apoE-independent.

To determine if exogenous apolipoproteins could serve as LPS acceptors during macrophage LPS efflux, we tested the activities of purified apoE and certain other exchangeable apolipoproteins that are normally found in HDL. As shown in Fig. 1D, low concentrations of exogenous apoE and apoC-I increased LPS efflux from THP-1 macrophages, whereas apoC-II was relatively ineffective at low concentrations. ApoA-I, the only apolipoprotein tested that is not expressed by macrophages, also promoted LPS efflux at low apolipoprotein concentrations. Although apoC1 mRNA was highly upregulated in cultured human macrophages (data not shown), apoC-I protein production by these cells was only 2–7% that of apoE (data not shown).

We next tested the hypothesis that ABCA1 plays a role in LPS efflux. The effects of agents that augment the expression of ABCA1 were compared in peritoneal macrophages from ABCA1$^{+/+}$ and ABCA1$^{-/-}$ mice. cAMP induces ABCA1 expression by an LXR-independent mechanism (Ref. 10 and data not shown), and T0901317 (48) is a nonsteroidal activator of the nuclear receptor LXR, which in turn activates ABCA1 transcription (8). The effects of these agents on ABCA1 expression are shown in the legend below supplementary Table II. To minimize the basal expression of ABCA1 and other LXR target genes, we first preincubated the macrophages in lipoprotein-deficient serum (i.e., serum that does not contain cholesterol or oxysterol ligands of LXR).

T0901317 increased LPS efflux in WT macrophages in SFM in the absence of exogenous acceptors (**Fig. 2A**), whereas it had little or no effect in macrophages that lacked ABCA1 (Fig. 2B). T0901317 also increased cholesterol efflux to an extent similar to that of LPS efflux in WT macrophages (Refs. 49, 50, and data not shown). T0901317 decreased the level of protease-sensitive (cell-surface) LPS in WT (Fig. 2C) but not in ABCA1$^{-/-}$ macrophages (Fig. 2D), and it decreased protease-resistant (predominantly

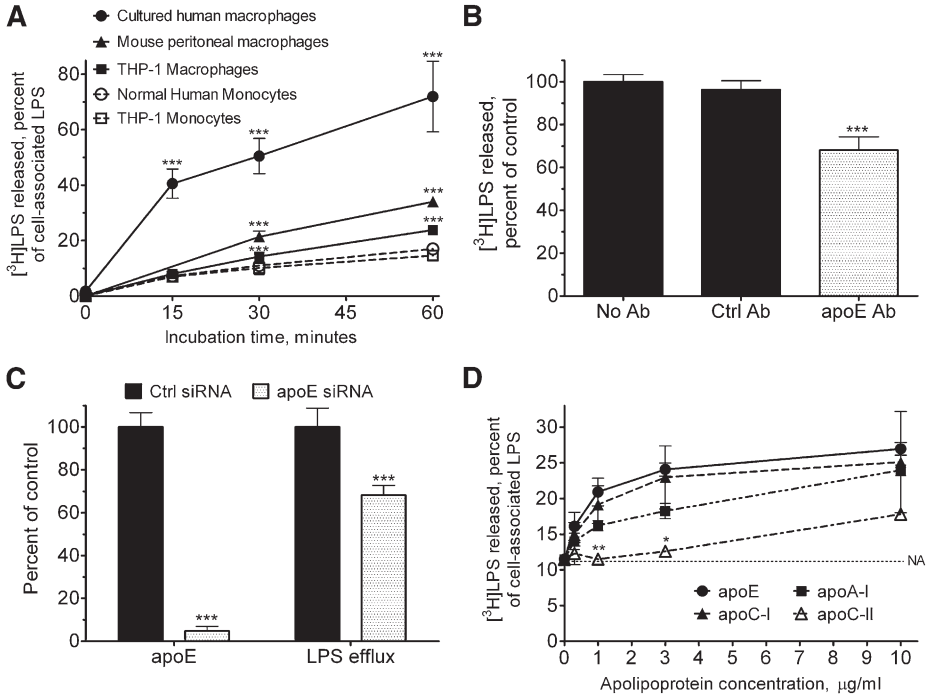

Fig. 1. Macrophage differentiation increases LPS efflux in human macrophages: role of apolipoproteins. A: The release of [^{3}H]LPS from the indicated cells into SFM-containing culture supernatants was measured after 15–60 min at 37°C as described in "Materials and Methods." Data from several experiments performed in duplicate are shown as mean ± 95% CI (n = 2–18 for each time point). Asterisks denote significant differences from monocytes of each cell type at each time point. B: The release of [^{3}H]LPS from cultured human macrophages was measured after 15 min in SFM in the presence of anti-apoE or control antibodies as described in "Materials and Methods." In two independent experiments, the control cells released 33% and 47% of their cell-associated LPS, respectively. Error bars denote mean ± SD. Asterisks denote significant differences from the no antibody (No Ab) (***P = 0.0001) and control antibody (Ctrl Ab) (***P = 0.0003) groups. C: ApoE expression in cultured human macrophages was inhibited by apoE siRNA as described in "Materials and Methods." ApoE protein was measured in the culture supernatants by ELISA, and [^{3}H]LPS efflux was measured as described in (B). The data are expressed as the percent of LPS or apoE released by the cells that received control siRNA (Ctrl siRNA). Data from two (LPS) or three (apoE) independent experiments are shown as mean ± SD. Asterisks denote significant differences from the controls (***P< 0.001). The control cells released 29% and 46% of their cell-associated LPS in the respective experiments. D: The release of [^{3}H]LPS from THP-1 macrophages was measured as described in "Materials and Methods" after incubating the cells for 30 min in SFM containing the indicated concentrations of purified apolipoproteins. Data from several experiments are shown as mean ± SD (n = 2–8 for each time point). Asterisks denote significant differences at each time point from cells that were incubated with apoE (*P< 0.05, **P< 0.01, ***P< 0.001). apo, apolipoprotein; LPS, lipopolysaccharide; SFM, serum-free medium; siRNA, small inhibitory RNA.

internalized) LPS after 60 min in WT macrophages (Fig. 2E) but not in ABCA1$^{-/-}$ macrophages (Fig. 2F). Under these lipoprotein-deficient conditions that minimize ABCA1 expression, the rate of LPS efflux in unstimulated (Ctrl) ABCA1$^{+/+}$ macrophages was similar to that of ABCA1$^{-/-}$ macrophages, suggesting the existence of an ABCA1-independent efflux mechanism. Nevertheless, the ABCA1$^{-/-}$ Ctrl group retained significantly more cell-surface

TABLE 1. Effects of human macrophage differentiation on apoE and ABC transporter gene expression

apoE mRNA	ABCA1 mRNA	ABCG1 mRNA	Cells
1.0 ± 0.8	1.00 ± 0.52	1.00 ± 0.96	Normal human monocytes
3.4 ± 1.1	0.05 ± 0.01	0.009 ± 0.002	THP-1 monocytes
52.6 ± 30[b]	0.23 ± 0.07[c]	0.008 ± 0.003	THP-1 macrophages
961.0 ± 538[b]	0.98 ± 0.29	0.077 ± 0.015[a]	Cultured human macrophages

Human monocytes were differentiated into macrophages in complete medium, and mRNA levels for apoE, ABCA1, and ABCG1 were measured by real time PCR as described in "Materials and Methods." The data are shown as fold-change from levels in normal human monocytes (mean ± SD, n = 6). Normal human monocytes and macrophages were derived from three healthy volunteers. ABCG1, ATP-binding cassette transporter G1; apo, apolipoprotein.

[a] P< 0.05 from the monocyte group of the same cell type.
[b] P< 0.01 from the monocyte group of the same cell type.
[c] P< 0.001 from the monocyte group of the same cell type.

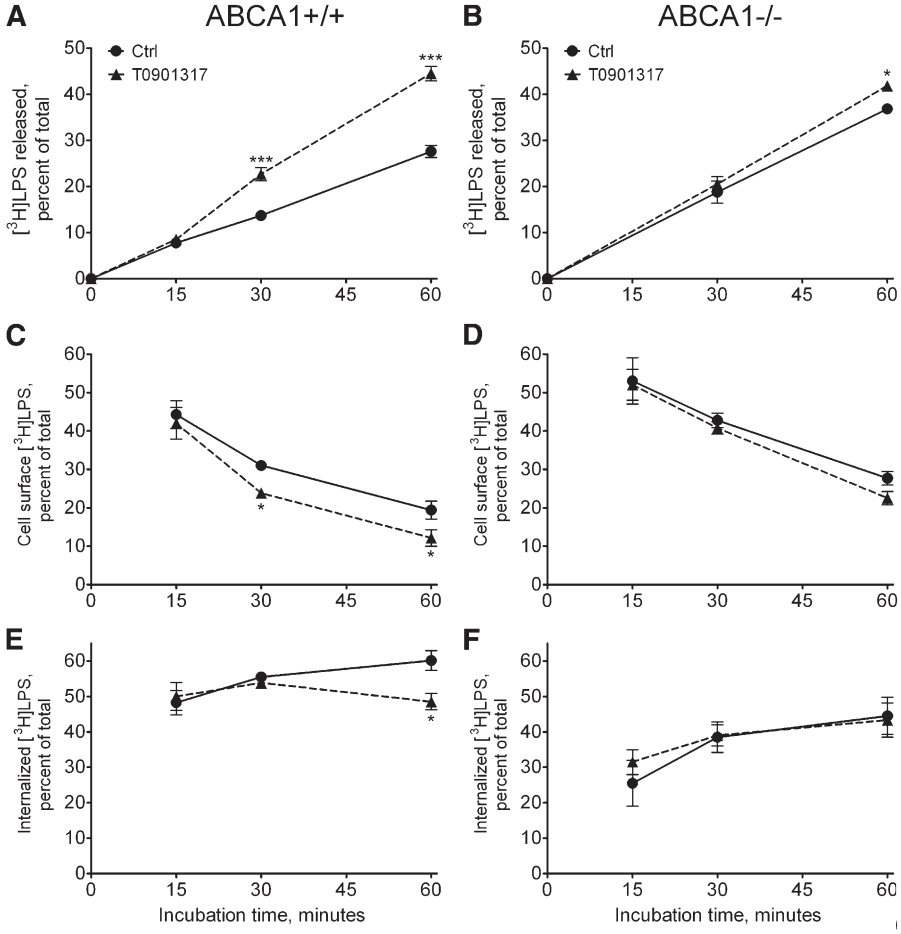

Fig. 2. ABCA1 promotes LPS efflux from macrophages. Peritoneal macrophages from WT ($ABCA1^{+/+}$) (A, C, E) and ABCA1-deficient ($ABCA1^{-/-}$) (B, D, F) mice were incubated for 16–24 h in medium containing lipoprotein-deficient FBS to minimize LXR activation in the control (Ctrl) group; the medium also contained 5 μM T0901317 or vehicle (Ctrl). A, B: LPS efflux was determined by measuring the release of [^{3}H]LPS into the culture supernatant as described in "Materials and Methods." Protease-resistant (cell-surface) [^{3}H]LPS (C, D) and protease-insensititve [^{3}H]LPS (principally internalized LPS) (E, F) were measured as described in "Materials and Methods." Data were compiled from several experiments and are shown as the percentage of the total initial cell-associated [^{3}H]LPS. Error bars denote mean ± SEM (n = 4–8). Asterisks denote significant differences from the controls (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). LPS, lipopolysaccharide; LXR, liver X receptor; WT, wild-type.

LPS than macrophages in the $ABCA1^{+/+}$ Ctrl group after 60 min (27.7 ± 4.4% versus 19.4 ± 6.5% (a 43% increase), $P = 0.02$) (Fig. 2C, D and supplementary Table II).

As shown in supplementary Table II, cAMP also increased LPS efflux in WT macrophages, but it had the reverse effect of slightly decreasing LPS efflux in $ABCA1^{-/-}$ macrophages. T0901317 had similar effects in the three WT mouse strains (DBA, C57Bl/6, and B6/129) used in these experiments. In $apoE^{-/-}$ mice, T0901317 induced less LPS efflux (17% increase) than in $apoE^{+/+}$ macrophages (41% increase), indicating the induction of an endogenous apoE-dependent efflux mechanism consistent with our results in human macrophages (Fig. 1B, C), suggesting a role for endogenous apoE. As expected, T0901317 had no effect on LPS efflux in $LXR\alpha\beta^{-/-}$ macrophages, indicating that the effects of this drug were mediated by LXR activation. The observed 60% increase in the basal level of LPS efflux in unstimulated $LXR\alpha\beta^{-/-}$ versus $LXR\alpha\beta^{+/+}$ macrophages may be due to increased basal ABCA1 expression in the $LXR\alpha\beta^{-/-}$ cells (8) accompanied by only a slight decrease in apoE expression (9).

We conclude from these experiments that ABCA1 and apoE promote LPS efflux from macrophages in the absence of an exogenously added LPS acceptor. Under preincubation conditions that minimize ABCA1 expression, the data also reveal LPS efflux mechanisms that are independent of ABCA1 or apoE in unstimulated $ABCA1^{-/-}$ or $apoE^{-/-}$ macrophages, respectively.

LPS induces ABCA1 expression and LPS efflux in the presence of exogenous apolipoproteins

To determine the effects of prior exposure of macrophages to LPS, we cultured $ABCA1^{+/+}$ and $ABCA1^{-/-}$ macrophages in the presence or absence of unlabeled LPS for 24 h in serum-containing medium, washed the cells, added [^{3}H]LPS, and performed LPS efflux assays in SFM in the presence or absence of apoE. The LPS pretreatment did not inhibit the binding of [^{3}H]LPS to the cells, and there

was no significant difference in LPS uptake (i.e., the total initial cell-associated LPS) between ABCA1$^{+/+}$ and ABCA1$^{-/-}$ macrophages (data not shown). As shown in **Fig. 3A**, naïve ABCA1$^{+/+}$ macrophages (preincubated without LPS) promoted significantly more [^{3}H]LPS efflux in 1 h than naïve ABCA1$^{-/-}$ macrophages, and exogenously added apoE increased LPS efflux slightly in cells from both strains. Preincubation of the macrophages with LPS decreased [^{3}H]LPS efflux in ABCA1$^{-/-}$ cells but not in ABCA1$^{+/+}$ cells. After LPS pretreatment, exogenous apoE induced a 77% increase in LPS efflux in control macrophages, whereas apoE had no effect in cells that lacked ABCA1. ApoA-I was similarly effective at promoting LPS efflux in ABCA1$^{+/+}$ cells that were preincubated with LPS (supplementary Fig. I).

The most striking effect of LPS preconditioning was its ability to prolong the retention of LPS on the cell surfaces of ABCA1$^{-/-}$ macrophages, whereas it decreased LPS retention on the surfaces of cells that expressed ABCA1 (Fig. 3B). The addition of apoE caused an additional decrease in cell-surface LPS in LPS-pretreated ABCA1$^{+/+}$ macrophages but had no effect on ABCA1$^{-/-}$ macrophages. Thus after LPS pretreatment, ABCA1$^{-/-}$ macrophages retained over 4-fold more cell-surface [^{3}H]LPS than ABCA1$^{+/+}$ macrophages after 1 h in the presence of apoE. Internalized (protease-resistant) [^{3}H]LPS was significantly decreased by LPS pretreatment only in ABCA1$^{-/-}$ macrophages (Fig. 3C). ApoE decreased internalized LPS in LPS-pretreated ABCA1$^{+/+}$ cells by nearly 50% but had no effect in cells that lacked ABCA1.

The ability of LPS preconditioning to augment the LPS efflux-promoting activity of apoE may be due to the ability of LPS to increase ABCA1 expression. Therefore, we tested the effects of LPS on macrophage expression of both ABCA1 and apoE. As shown in **Table 2**, exposure of macrophages to LPS for 16–24 h increased ABCA1 mRNA levels over 5-fold, whereas apoE mRNA was decreased by 43% and was suppressed somewhat more after 48 h; T0901317 increased ABCA1 mRNA to high levels, as expected. As shown in **Fig. 4**, Western blots of the total membrane fraction of macrophages showed that LPS induced a striking increase in ABCA1 protein by 24 h, which decreased only slightly after 48 h. As expected, LPS pretreatment decreased apoE secretion by over 60% (Table 2), and it also decreased cellular apoE in macrophage membrane fractions (Fig. 4A, B).

We conclude that after exposure of macrophages to LPS, ABCA1-deficient macrophages retain LPS on the cell surface for a longer period of time than WT macrophages. Moreover, LPS preconditioning appears to decrease the activity of ABCA1-independent LPS efflux mechanisms, and that preconditioning makes apoE-mediated LPS efflux completely dependent upon ABCA1. The data also suggest that the ability of LPS to induce ABCA1 and to suppress apoE expression both contribute to the enhanced ability of exogenous apolipoproteins to promote LPS efflux in LPS-pretreated WT macrophages.

Macrophages rapidly internalize LPS and retain it for an extended period of time (28, 47). To determine whether

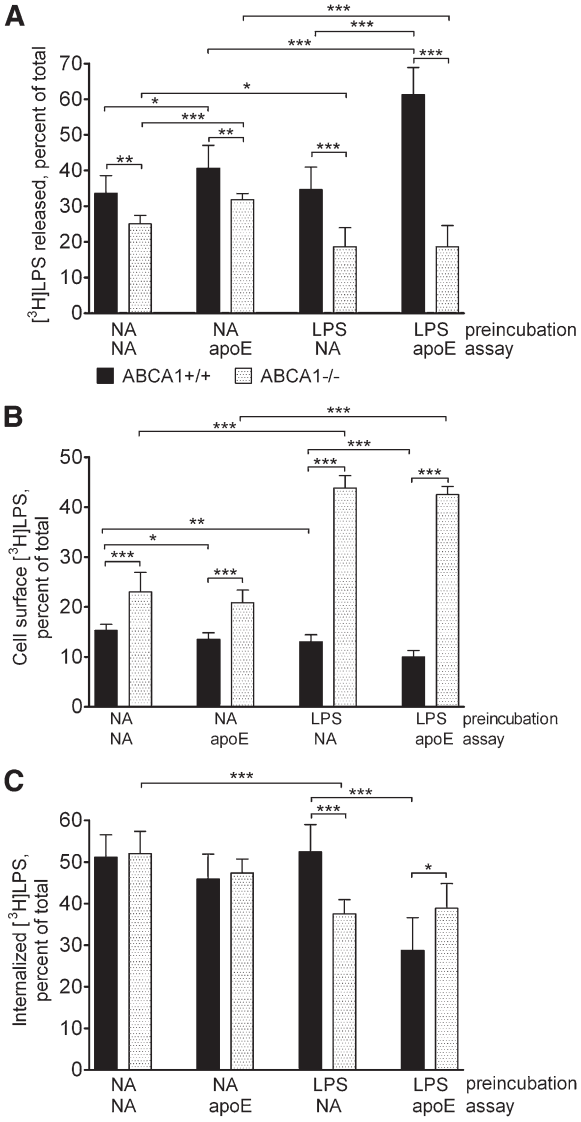

Fig. 3. LPS preconditioning of macrophages increases ABCA1-dependent LPS efflux into exogenous apolipoproteins. Peritoneal macrophages from ABCA1$^{+/+}$ and ABCA1$^{-/-}$ mice were preincubated for 24 h in complete medium containing 10% FBS without (NA) or with unlabeled LPS (preincubation). The cells were allowed to take up [^{3}H]LPS for 10 min in the presence of 10% FBS and were then washed and incubated in SFM with or without apoE (5 μg/ml) for 60 min (assay). (A) LPS efflux, (B) cell-surface LPS, and (C) internalized LPS were measured as described in Fig. 2 and expressed as percentage of the total initial cell-associated LPS. Error bars denote mean ± SD for 3–4 experiments performed in duplicate. Asterisks denote significant differences as indicated (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). apo, apolipoprotein; LPS, lipopolysaccharide; SFM, serum-free medium.

ABCA1 can remove LPS long after it has been taken up by macrophages, we loaded WT and ABCA1$^{-/-}$ macrophages with radiolabeled LPS for 24 h, washed the cells, and measured cellular and effluxed LPS daily for up to six days. We used a [^{3}H/^{14}C]LPS preparation in which each of the fatty acyl chains was labeled with ^{3}H and the glucosamine backbone of lipid A was labeled with ^{14}C. As shown in supplementary Fig. IIA, LPS efflux was significantly increased in ABCA1$^{+/+}$ macrophages compared with that of ABCA1$^{-/-}$

TABLE 2. LPS induces ABCA1 and suppresses apoE expression in macrophages

Macrophages	Incubation Time	ABCA1 mRNA	ABCG1 mRNA	ApoE mRNA	ApoE Secreted
Ctrl	24 or 48 h	1.0 ± 0.05	1.0 ± 0.09	1.0 ± 0.07	1.00 ± 0.06
LPS	16–24 h	5.3 ± 1.1[b]	0.31 ± 0.16[a]	0.57 ± 0.07[b]	0.34 ± 0.04[b]
LPS	48 h	ND	ND	0.39 ± 0.16[b]	0.41 ± 0.06[b]
T	24 h	35.1 ± 11[b]	ND	ND	ND

Peritoneal macrophages from wild-type mice were incubated as described in Fig. 3 in the presence or absence of LPS or 5 μM T0901317 (T) for the indicated times. mRNA levels were measured by real time PCR as described in "Materials and Methods." To measure secreted apoE, the medium was replaced with fresh medium after the indicated incubation times, and apoE was measured after 8 h in the culture supernatants by Western blotting. The data are shown as fold-change from levels in unstimulated control macrophages (Ctrl) and are expressed as mean ± SD for mRNA (n = 5) and secreted apoE (n = 3). ABCG1, ATP-binding cassette transporter G1; apo, apolipoprotein; Ctrl, unstimulated control macrophages; LPS, lipopolysaccharide; T, T0901317; ND, not done.

[a] $P < 0.01$ from Ctrl.

[b] $P < 0.001$ from Ctrl.

macrophages. After LPS loading at the beginning of the efflux time course (time 0), only 14% of the total cellular [^{3}H/^{14}C]LPS could be removed from the cell surface by protease treatment, suggesting that the remaining ~86% of the cellular LPS had been internalized. After three days, the ABCA1$^{+/+}$ cells had released approximately 40% of the cell-associated LPS into the culture medium, whereas ABCA1$^{-/-}$ cells release approximately 30%. Throughout days 1–6, very little (~5%) of the total LPS could be identified on the cell's surfaces. We conclude that although ABCA1 appears to have a more robust role in removing LPS from the cell surface (Fig. 3), the data also suggest that ABCA1 plays a significant role in the efflux of intracellular LPS (supplementary Fig. IIA). Similar results were obtained using a smooth-form [^{3}H/^{14}C]LPS in which the ^{3}H was in the polysaccharide chain and not in the fatty acyl chains (data not shown).

The intracellular enzyme AOAH partially deacylates LPS over a period of days by removing two of its six secondary fatty acyl chains, a process that greatly decreases LPS bioactivity (27, 28). To determine the extent of LPS deacylation during the six-day efflux time course, we measured the ^{3}H to^{14}C ratio of the ethanol-precipitable material in both cells and culture supernatants. As shown in supplementary Fig. IIB, immediately after the 24 h [^{3}H/^{14}C]LPS loading period, only approximately 20% of the secondary fatty acyl chains had been removed. Deacylation increased after one additional day. However, the loss of secondary fatty acyl chains from the LPS did not exceed approximately 50% during the six-day period, suggesting that a significant amount of fully acylated bioactive LPS was inaccessible to AOAH in both ABCA1$^{+/+}$ and ABCA1$^{-/-}$ macrophages. These results support the hypothesis that ABCA1 plays a role in removing both bioactive (fully acylated) and attenuated or inactive (partially deacylated) LPS from macrophages.

Delayed recovery from LPS-induced tolerance in ABCA1-deficient macrophages

To determine how prolonged retention of LPS by ABCA1$^{-/-}$ macrophages affects cell function, we measured the ability of macrophages to recover from LPS-induced tolerance after preincubation with LPS for 24 h. Using real time PCR, we measured steady-state mRNA levels of a panel of ten LPS-responsive genes. After LPS preconditioning, some of these genes became hyporesponsive to subsequent LPS challenge (**Fig. 5A**, **Fig. 6A**, and supplementary Table III), and the other genes either responded normally to subsequent LPS challenge or were "primed" and hyper-responsive to subsequent LPS challenge (Fig. 5B, Fig. 6B, and supplementary Table III). The responsiveness of the nontolerizable genes is due to chromatin modifications induced during LPS preincubation, which render these genes permissive for response to LPS (26). The analysis of IL-1β, a representative tolerizable gene (Fig. 5A), shows that LPS induced similar IL-1β mRNA levels in ABCA1$^{+/+}$ and ABCA1$^{-/-}$ macrophages that had not been preincubated with LPS. In contrast, cells that received a

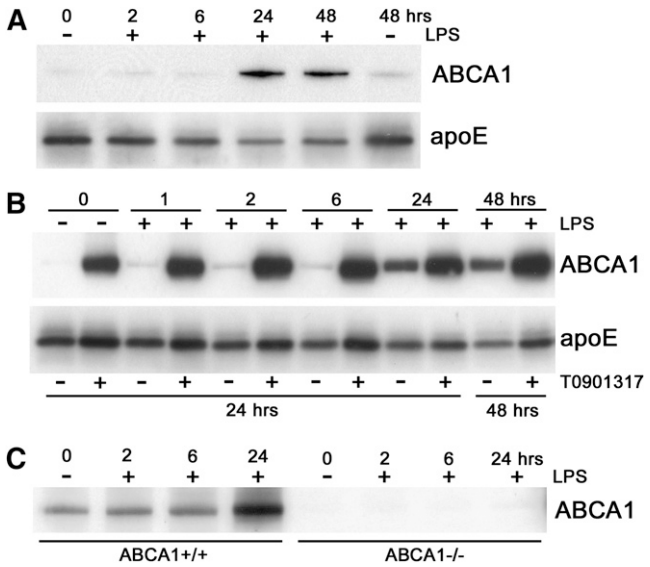

Fig. 4. LPS preconditioning of macrophages increases cellular ABCA1 and decreases cellular apoE. Peritoneal macrophages from ABCA1$^{+/+}$ (A, B, C) and (ABCA1$^{-/-}$) (C) mice were cultured overnight in medium containing 10% FBS in the presence or absence of 5 μM T0901317, where indicated, followed by the addition of LPS (10–100 ng/ml) for the indicated times. Total membrane fractions were prepared from cell lysates, and equal amounts of membrane protein were run on SDS-PAGE gels. The proteins were transferred and immunoblotted with antibodies to ABCA1 or apoE as described in "Materials and Methods." Each panel represents a separate experiment derived from WT C57Bl/6 macrophages (A, B) and B6:129Sv ABCA1$^{+/+}$ and ABCA1$^{-/-}$ macrophages (C). apo, apolipoprotein; LPS, lipopolysaccharide; WT, wild-type.

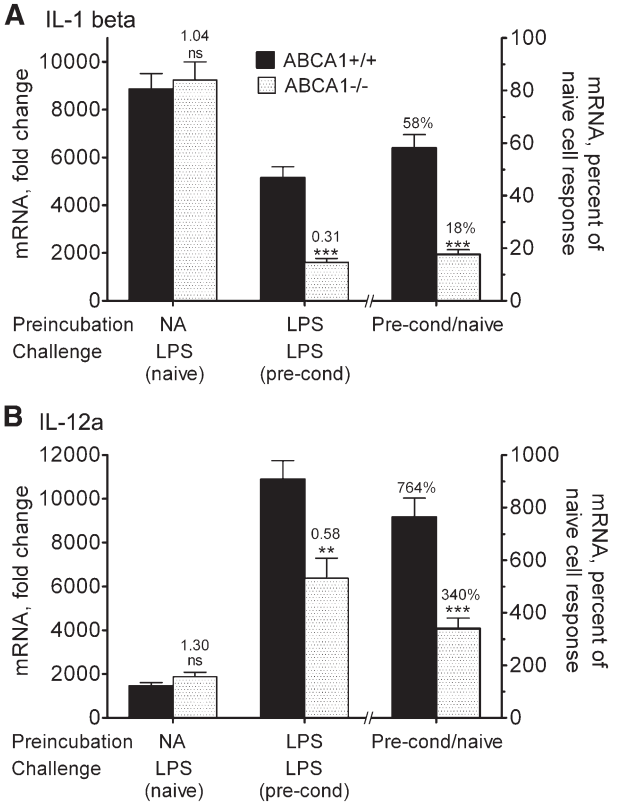

Fig. 5. Diminished mRNA responses in LPS-challenged ABCA1$^{-/-}$ macrophages after LPS preconditioning. ABCA1$^{+/+}$ and ABCA1$^{-/-}$ macrophages were preincubated in complete medium with (pre-cond) or without (naïve) 10 ng/ml LPS for 24 h as described in "Materials and Methods." After washing, the cells were challenged with 10 ng/ml LPS for 2 h. mRNA levels were measured by real time PCR and normalized with 18S rRNA as described in "Materials and Methods." Data for IL-1β (A) and IL-12a (B) are expressed as the fold change from the unchallenged naïve ABCA1$^{+/+}$ cells. Ratios (ABCA1$^{-/-}$ to ABCA1$^{+/+}$) of mRNA levels are shown above the bars. The responses of the LPS-preconditioned cells expressed as percentages of the naïve cell responses are shown on the right of each graph (Pre-cond/naïve). Error bars represent mean ± SEM of three experiments performed in duplicate representing a total of six ABCA1$^{+/+}$ and seven ABCA1$^{-/-}$ animals. Significant differences between ABCA1$^{-/-}$ and ABCA1$^{+/+}$ groups are denoted (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). IL, interleukin; LPS, lipopolysaccharide.

tolerizing dose of LPS for 24 h had a diminished response to subsequent LPS challenge. The response to LPS challenge was significantly more diminished in cells that lacked ABCA1 both in terms of fold change from unstimulated control macrophages (0.31 ratio of ABCA1$^{-/-}$ to ABCA1$^{+/+}$) and percentage of mRNA levels in LPS-challenged naïve macrophages that had not been pretreated with LPS (58% in ABCA1$^{+/+}$ versus 18% in ABCA1$^{-/-}$ cells). The analysis of IL-12a, a representative nontolerizable gene (Fig. 5B), shows that LPS challenge induced similar mRNA levels in naïve ABCA1$^{+/+}$ and ABCA1$^{-/-}$ macrophages, whereas LPS preconditioning resulted in an increased response to LPS challenge in both groups. However, the response to LPS was significantly diminished in ABCA1$^{-/-}$ macrophages as expressed by both the fold change of mRNA level from the unstimulated control (0.58 ratio of ABCA1$^{-/-}$ to ABCA1$^{+/+}$) and the percentage of mRNA levels in naïve cells (764% in ABCA1$^{+/+}$ versus 340% in ABCA1$^{-/-}$ cells). Similar results were obtained with most of the other genes analyzed, as shown in Fig. 6 (day 0) and supplementary Table III (day 0).

To measure the recovery of macrophages from tolerance, we incubated naïve or LPS-conditioned cells in fresh medium for 1–5 days and subsequently challenged them with LPS for 2 h. Fig. 6 shows mRNA levels after LPS challenge of LPS-conditioned macrophages expressed as the percentage of mRNA levels in LPS-challenged naïve macrophages. Thus, a level of 100% would indicate a return to normal responsiveness for each experimental group. The results show that, for tolerizable genes, ABCA1$^{-/-}$ macrophages generally had lower responses after LPS-conditioning and delayed recovery from tolerance compared with ABCA1$^{+/+}$ macrophages (Fig. 6A). Nontolerizable genes also showed decreased LPS responsiveness in ABCA1$^{-/-}$ macrophages during the same period after LPS conditioning (Fig. 6B). Differences were most pronounced after one day of recovery, at which time seven genes showed significantly lower responsiveness to LPS challenge in ABCA1$^{-/-}$ macrophages. After two days of recovery, four genes remained significantly less responsive in the ABCA1$^{-/-}$ group. After five days of recovery, three of the tolerizable genes (TNF, IL-1β, and IL-12b) had not fully recovered to normal responsiveness in both groups, and the most profoundly tolerized gene, IL-12b, remained more suppressed in the ABCA1$^{-/-}$ group. Similar results were obtained for each time point when the data were expressed as fold changes from unstimulated naïve macrophages (supplementary Table III).

To analyze the recovery from tolerance by measuring cytokine protein production, we challenged naïve or LPS-conditioned macrophages with LPS in the same manner as the above mRNA experiments, except that the challenge was continued for 6 h to allow time for protein production of TNF, IL-6, and RANTES, which were measured in the culture supernatants by ELISA. As shown in **Fig. 7A**, TNF was profoundly tolerized in both ABCA1$^{+/+}$ and ABCA1$^{-/-}$ macrophages. However, the ABCA1$^{-/-}$ cells remained less responsive to LPS than the ABCA1$^{+/+}$ cells throughout the five-day recovery period. The ABCA1$^{+/+}$ cells fully regained their ability to produce IL-6 after one day of recovery, whereas the ABCA1$^{-/-}$ cells remained tolerant; although both groups of macrophages were equally responsive to LPS challenge after two days of recovery, the ABCA1$^{+/+}$ group became significantly primed after five days, whereas the responsiveness of the ABCA1$^{-/-}$ did not change (Fig. 7B). In contrast, both groups of macrophages produced slightly more RANTES when challenged immediately after LPS conditioning (day 0). After one day of recovery, the ABCA1$^{-/-}$ cells became significantly hyporesponsive for RANTES compared with the ABCA1$^{+/+}$ group and remained so during the five days of recovery. When the data were expressed as total cytokine production (ng/100 μg cell protein) in LPS-conditioned macrophages (supplementary Fig. IIID-F), TNF production was almost completely ablated after LPS challenge in ABCA1$^{-/-}$ cells and remained so after two days of recovery; after five days of recovery, the response of the ABCA1$^{-/-}$ cells was still

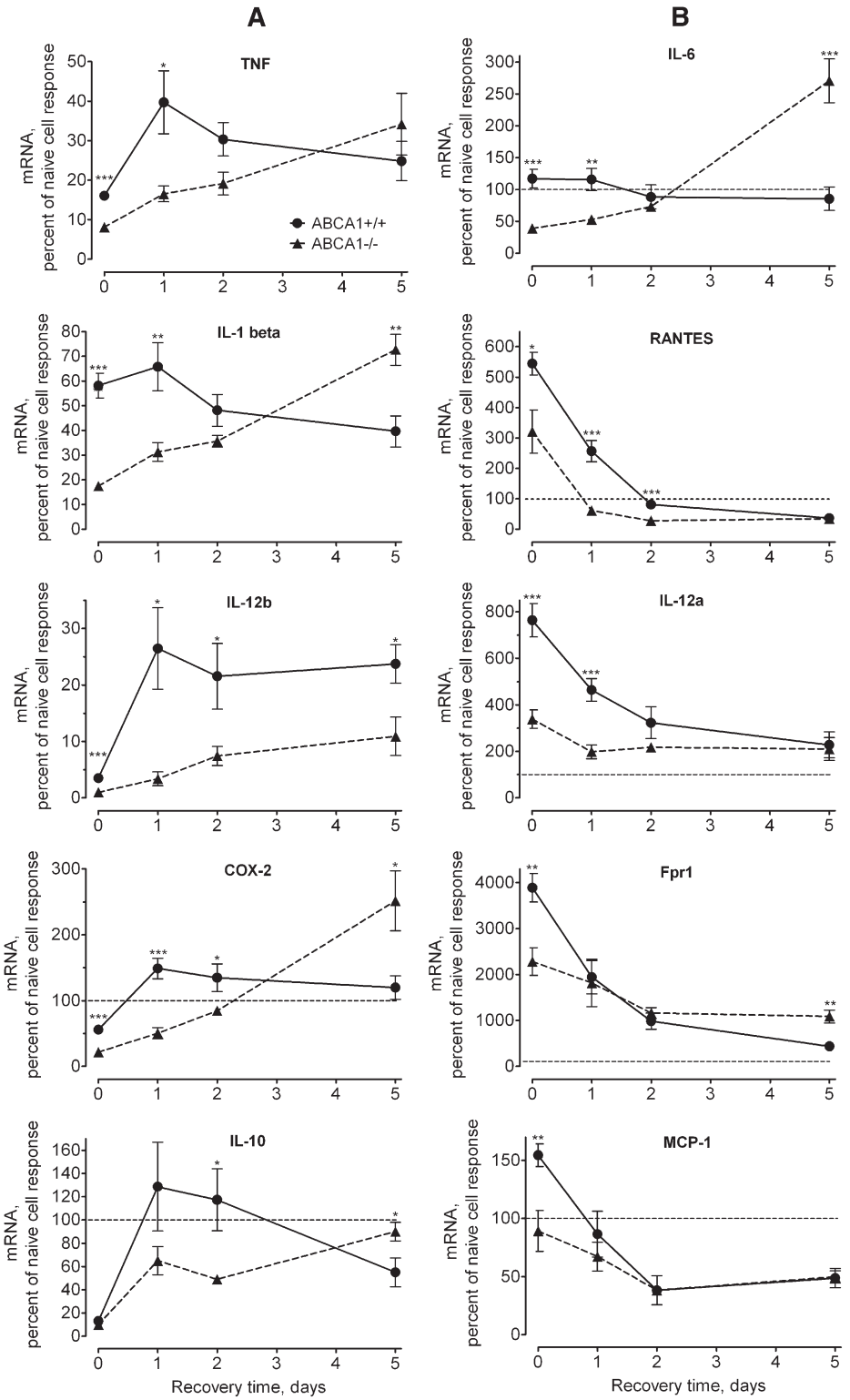

Fig. 6. Delayed recovery from LPS-induced tolerance in ABCA1-deficient macrophages (mRNA responses). To measure the time course of recovery of LPS responsiveness after preconditioning, the experiments were performed as described in Fig. 5, except that the cells were washed twice after the 24 h LPS preincubation and incubated in fresh medium for 0–5 days before LPS challenge. The responses of the LPS-preconditioned cells are expressed as percentages of the naïve cell responses (Pre-cond/naïve) are represented as described in Fig. 5. Genes that were tolerized on day 0 are shown in column A, and nontolerized genes are shown in column B. COX-2, cyclooxygenase-2; Fpr1, formyl peptide receptor 1; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; TNF, tumor necrosis factor.

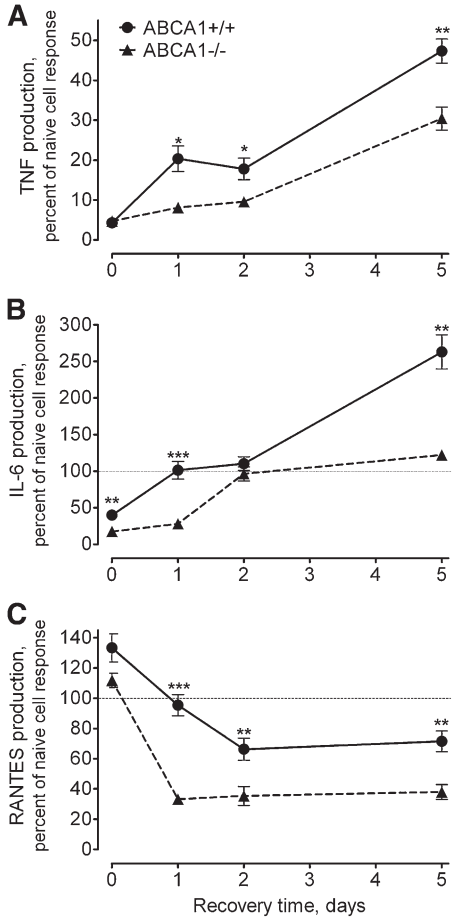

Fig. 7. Delayed recovery from LPS-induced tolerance in ABCA1-deficient macrophages (cytokine production). ABCA1$^{+/+}$ and ABCA1$^{-/-}$ macrophages were preincubated for 24 h with or without LPS as described in Fig. 5, washed twice, incubated in fresh complete medium for 0–5 days, and challenged with 10 ng/ml LPS for 6 h. TNF (A), IL-6 (B), and RANTES (C) were measured in the culture supernatants by ELISA as ng/100 μg total cellular protein. The responses of the LPS-preconditioned cells are expressed as percentages of the naïve cell responses. The ABCA1$^{+/+}$ data were derived from 15 determinations from a total of 11 mice, and the ABCA1$^{-/-}$ data were from 8 determinations from 8 mice (cells from more than one mouse were mixed in some experiments). Error bars denote mean ± SEM. Asterisks denote significant differences between the groups at each time point (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

significantly decreased below that of ABCA1$^{+/+}$ cells. Likewise, RANTES production was significantly reduced in LPS-conditioned ABCA1$^{-/-}$ macrophages throughout the five-day recovery period, whereas IL-6 production was reduced only on days 0 and 1. In naïve macrophages (supplementary Fig. IIIA-C), RANTES production was the same in both ABCA1 groups through day 5, and IL-6 production was not significantly different through day 2. However, naïve ABCA1$^{-/-}$ cells produced more IL-6 than ABCA1$^{+/+}$ cells on day 5, which explains the lack of priming for IL-6 production in the ABCA1$^{-/-}$ cells (Fig. 7B). Unexpectedly, TNF production was significantly decreased in naïve ABCA1$^{-/-}$ cells through day 2 (supplementary Fig. IIIA) in contrast to IL-6 and RANTES, which were not significantly different.

In summary, the data show that LPS-conditioned ABCA1$^{-/-}$ macrophages were hyporesponsive to LPS in their ability to produce TNF and RANTES protein throughout the five-day recovery period, whereas the cells regained their ability to produce normal levels of IL-6 after two days of recovery. Although differences in responses to LPS challenge between LPS-preconditioned ABCA1$^{+/+}$ and ABCA1$^{-/-}$ macrophages showed a great deal of gene-specific variation in mRNA and protein levels, the ABCA1$^{-/-}$ cells showed a general pattern of delayed recovery from tolerance and/or decreased responsiveness to LPS challenge compared with ABCA1$^{+/+}$ cells.

DISCUSSION

Our data show for the first time that ABCA1 plays significant roles in both the efflux of bacterial LPS from macrophages and in promoting macrophage recovery from LPS-induced tolerance. The data also show that endogenous apoE can serve as an LPS acceptor during LPS efflux in resting macrophages, whereas exogenous apolipoproteins (e.g., apoA-I and apoE) serve this function during macrophage activation (i.e., LPS preconditioning), a state in which endogenous apoE production is suppressed and ABCA1 expression is induced.

Although reports of the effects of LPS on macrophage ABCA1 expression are conflicting (14–18), our experiments consistently showed that LPS increased ABCA1 expression at the mRNA and protein levels in peritoneal macrophages. In keeping with a previous report (14), our findings that LPS also inhibited the expression of the LXR target genes ABCG1 and apoE under the same conditions suggest that the positive effects of LPS on ABCA1 expression occurred through an LXR-independent pathway. We also found that LPS administration to WT mice increased ABCA1 mRNA levels in the liver (R. L. Kitchens, unpublished observations) as previously reported (14). Another study showed that TNF also increased ABCA1 expression in peritoneal macrophages (13), whereas it had the opposite effect in the J774 macrophage cell line (16). Others found that LPS decreased the expression of ABCA1 in RAW (15) and J774 (16) macrophage cell lines. In other macrophage studies, the inhibition of ABCA1 expression by LPS required the activation of interleukin-1 receptor associated kinase (IRAK)-1 (18), and Tlr4 and Tlr3 agonists inhibited the expression of ABCA1 and other LXR target genes by inhibiting LXR activation by an interferon regulatory factor (IRF)3-dependent mechanism, whereas TNF and Tlr2 signaling had no effects (17). The reasons for these divergent findings in the literature are unclear. One potential factor may be related to the effects of lipids in the macrophage culture medium. The studies that show increased ABCA1 expression in response to LPS (14) and TNF (13) were performed in the presence of serum (0.5–10% FBS), whereas those that show LPS-induced inhibition of ABCA1 expression were performed under serum-free conditions (15, 16), in medium containing lipoprotein-deficient serum (17), or under low (1%) serum conditions (18). Our

experiments were performed in the presence of 10% FBS. However, in two of our preliminary experiments performed in medium containing lipoprotein-deficient serum, we did not see an increase in macrophage ABCA1 protein levels in response to LPS (data not shown). Considerably more work will be required to establish whether lipid signaling can promote the ability of LPS to induce ABCA1 expression. Other potential factors may include differences in macrophage subpopulations, which may express ABCA1 either constitutively or inducibly (51).

Prior exposure of macrophages to LPS reduced the activity of one or more ABCA1-independent LPS efflux mechanisms (Fig. 3), but their identities remain unclear. Although we cannot rule out the possibility that ABCG1 plays a role in LPS efflux, our measurements of ABCG1 mRNA levels under certain conditions do not point to a role for this protein (Tables 1 and 2). We also considered the possibility that rapid LPS release from human macrophages could be due to CD14 shedding from the membrane. However, flow cytometry analysis of the macrophages showed no decrease in mCD14 density after LPS efflux, and the effluxed LPS could not be immunoprecipitated by anti-CD14 antibodies that were immobilized on agarose beads (data not shown), suggesting that CD14 shedding did not play a significant role. We also found that rapid LPS efflux occurred regardless of the physical state of the LPS that was bound to mCD14 (i.e., LPS monomers versus aggregates (47)), suggesting that LPS efflux was not due to LPS release from LPS aggregates on the cell surface (data not shown).

The degree of ABCA1-dependency of LPS efflux appears to vary with the activation state of the macrophage. In unstimulated macrophages, the rates of LPS efflux in cells that lacked ABCA1 or apoE were similar to those of the WT controls, although the $ABCA1^{-/-}$ cells retained more LPS on their surfaces than $ABCA1^{+/+}$ cells (Fig. 2D, C; Fig. 3B; and supplementary Table II). However, the rates of LPS efflux increased in the WT controls upon stimulation with agents that increased ABCA1. The unstimulated baseline efflux rates may be similar due to low ABCA1 expression in the WT macrophages and possibly to the induction of a compensatory phenotype in $ABCA1^{-/-}$ or $apoE^{-/-}$ cells in which redundant efflux mechanisms may be increased. However, after LPS preconditioning, LPS efflux became more dependent upon both ABCA1 and exogenous LPS acceptors (e.g., apoA-I and apoE) (Fig. 3). This was due, in part, to the ability of LPS to suppress endogenous apoE expression and to induce ABCA1, and it may also be due to the suppression of one or more redundant LPS efflux mechanisms. Under these conditions, exogenous apoE had no ability to increase LPS efflux in macrophages that lacked ABCA1, and these cells retained LPS on their surfaces even in the presence of apoE (Fig. 3B).

These data suggest that ABCA1 can efficiently remove cell-surface LPS from LPS-preconditioned cells over relatively short periods of time (e.g., 1 h). However, the results of LPS preconditioning experiments in which the cells were loaded with radiolabeled LPS for 24 h (supplementary Fig. II) suggest that ABCA1 also promotes the efflux of internalized LPS over a period of days. During the six-day time course of these experiments, very little (~5%) of the total LPS could be identified on the cell surface, although we cannot rule out the possibility that some of the protease-resistant LPS may be inserted into the cell membrane or bound to a proteinase K-resistant surface protein. It is unclear whether ABCA1 transports LPS from intracellular compartments or if ABCA1 removes LPS from the cell surface as it gradually recycles from intracellular compartments; the former mechanism is suggested by the lack of accumulation of cell-surface LPS in $ABCA1^{-/-}$ macrophages under these conditions. As the LPS efflux assays in this study measure the bulk flow of cell-associated LPS, they cannot account for the amounts of LPS that move from specialized microenvironments that may exist in the cell surface or intracellular compartments which may or may not contribute to Tlr4 signaling (52). Therefore, it is difficult to hypothesize how much of the total LPS must be removed from macrophages to reverse the LPS-induced attenuation of Tlr4 signaling. These experiments also showed that only half maximal deacylation of secondary fatty acyl chains had occurred even by day 6 in both cell-associated and effluxed LPS, suggesting that ABCA1 promotes the efflux of both fully and partially deacylated LPS.

Our data show that LPS-induced tolerance occurred to a greater degree in macrophages that lacked ABCA1 and that these cells were delayed in their recovery from the tolerant state. We hypothesize that this is due to decreased LPS efflux in LPS-preconditioned $ABCA1^{-/-}$ macrophages, although we cannot formally rule out other explanations. Differences in tolerance between $ABCA1^{-/-}$ and $ABCA1^{+/+}$ macrophages were not due to differences in the initial responses of the naïve macrophages to LPS. We consistently found that initial responses of $ABCA1^{-/-}$ cells to LPS was the same as those of $ABCA1^{+/+}$ cells for almost every gene that we tested (supplementary Table III and Fig. IIIA-C). These findings are similar to a previous report (5) in which LPS response differences were rarely significant between $ABCA1^{-/-}$ and $ABCA1^{+/+}$ macrophages. Others have reported modest but significant increases in $ABCA1^{-/-}$ macrophage responses to LPS (4, 6), but the basis for these conflicting findings in the literature remains unclear.

Our hypothesis that ABCA1-mediated LPS efflux is responsible for the rapid recovery from tolerance is also supported by the observation that significant differences between the response to LPS challenge in $ABCA1^{-/-}$ and $ABCA1^{+/+}$ macrophages always reveal a lower response in $ABCA1^{-/-}$ cells during the tolerant state in all genes tested. Even nontolerizable genes that were initially primed by preexposure to LPS (e.g., RANTES, IL-12a, and Fpr1) also showed decreased responses to LPS challenge in $ABCA1^{-/-}$ cells. These results are consistent with findings in $AOAH^{-/-}$ macrophages in which LPS remains bioactive for weeks; in these experiments, both nontolerizable genes (e.g., RANTES and IL-1RA) and tolerizable genes were hyporesponsive to LPS challenge in LPS-preconditioned

AOAH$^{-/-}$ macrophages (28). Although LPS-induced chromatin modifications in nontolerizable genes make them permissive for responsiveness to LPS, prolonged exposure to LPS may decrease their responsiveness to LPS challenge by negative feedback mechanisms that attenuate but do not ablate Tlr4 signaling (24). Taken together, our data are consistent with the hypothesis that prolonged interaction of LPS with Tlr4 in ABCA1$^{-/-}$ macrophages attenuates Tlr4 signal responses during the state of LPS-induced tolerance and gene reprogramming.

Our findings raise the possibility that macrophage expression of ABCA1 may play a physiologically significant role in live animals by removing LPS from macrophages and helping to restore normal innate immune responsiveness. By accelerating the recovery of macrophages from the tolerant state, ABCA1 may help to limit the period of time that animals are immunosuppressed and more vulnerable to further attack by pathogens after an initial encounter with Gram-negative (LPS-bearing) bacteria. Although increased infection rates have not been described in patients with Tangier disease, the rarity of this genetic disorder would make this very difficult to measure. ABCA1 may also speed the recovery of macrophages from tolerance during noninfectious conditions that involve either chronic exposure to endotoxin or gut translocation of endotoxin; these conditions may include smoking (53), trauma and surgery (25), hepatic cirrhosis (54), HIV infection (55), and asthma (56). As LPS is environmentally ubiquitous and gut translocation of LPS occurs normally at low levels, ABCA1 may also be instrumental in maintaining normal macrophage responsiveness in healthy individuals by keeping macrophages clear of bioactive LPS. Confirmation of the in vivo significance of this novel role of ABCA1 awaits carefully controlled studies in experimental animals.

The authors thank Dr. David Mangelsdorf for critically reading the manuscript, for providing mice and reagents, for advice and helpful discussions, and for generously providing the assistance of our collaborator and coauthor, Karine Gauthier, during her time in the Mangelsdorf Lab as postdoctoral Research Associate in the Howard Hughes Medical Institute. We also thank Dr. Robert Munford for providing LPS, for advice and helpful discussions, and for critically reading the manuscript. We thank Dr. Mingfang Lu for helpful discussions.

REFERENCES

1. Tang, C., and J. F. Oram. 2009. The cell cholesterol exporter ABCA1 as a protector from cardiovascular disease and diabetes. *Biochim. Biophys. Acta.* **1791:** 563–572.
2. Yvan-Charvet, L., N. Wang, and A. R. Tall. 2010. Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses. *Arterioscler. Thromb. Vasc. Biol.* **30:** 139–143.
3. Francone, O. L., L. Royer, G. Boucher, M. Haghpassand, A. Freeman, D. Brees, and R. J. Aiello. 2005. Increased cholesterol deposition, expression of scavenger receptors, and response to chemotactic factors in Abca1-deficient macrophages. *Arterioscler. Thromb. Vasc. Biol.* **25:** 1198–1205.
4. Koseki, M., K. I. Hirano, D. Masuda, C. Ikegami, M. Tanaka, A. Ota, J. C. Sandoval, Y. Nakagawa-Toyama, S. B. Sato, T. Kobayashi, et al. 2007. Increased lipid rafts and accelerated lipopolysaccharide-induced tumor necrosis factor-{alpha} secretion in Abca1-deficient macrophages. *J. Lipid Res.* **48:** 299–306.
5. Yvan-Charvet, L., C. Welch, T. A. Pagler, M. Ranalletta, M. Lamkanfi, S. Han, M. Ishibashi, R. Li, N. Wang, and A. R. Tall. 2008. Increased inflammatory gene expression in ABC transporter-deficient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions. *Circulation.* **118:** 1837–1847.
6. Zhu, X., J-Y. Lee, J. M. Timmins, J. M. Brown, E. Boudyguina, A. Mulya, A. K. Gebre, M. C. Willingham, E. M. Hiltbold, N. Mishra, et al. 2008. Increased cellular free cholesterol in macrophage-specific Abca1 knock-out mice enhances pro-inflammatory response of macrophages. *J. Biol. Chem.* **283:** 22930–22941.
7. Baldan, A., A. V. Gomes, P. Ping, and P. A. Edwards. 2008. Loss of ABCG1 results in chronic pulmonary inflammation. *J. Immunol.* **180:** 3560–3568.
8. Repa, J. J., S. D. Turley, J-M. A. Lobaccaro, J. Medina, L. Li, K. Lustig, B. Shan, R. A. Heyman, J. M. Dietschy, and D. J. Mangelsdorf. 2000. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science.* **289:** 1524–1529.
9. Laffitte, B. A., J. J. Repa, S. B. Joseph, D. C. Wilpitz, H. R. Kast, D. J. Mangelsdorf, and P. Tontonoz. 2001. LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. *Proc. Natl. Acad. Sci. USA.* **98:** 507–512.
10. Oram, J. F., R. M. Lawn, M. R. Garvin, and D. P. Wade. 2000. ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J. Biol. Chem.* **275:** 34508–34511.
11. Rubic, T., and R. L. Lorenz. 2006. Downregulated CD36 and ox-LDL uptake and stimulated ABCA1/G1 and cholesterol efflux as anti-atherosclerotic mechanisms of interleukin-10. *Cardiovasc. Res.* **69:** 527–535.
12. Panousis, C. G., G. Evans, and S. H. Zuckerman. 2001. TGF-{beta} increases cholesterol efflux and ABC-1 expression in macrophage-derived foam cells: opposing the effects of IFN-{{gamma}}. *J. Lipid Res.* **42:** 856–863.
13. Gerbod-Giannone, M. C., Y. Li, A. Holleboom, S. Han, L. C. Hsu, I. Tabas, and A. R. Tall. 2006. TNF{alpha} induces ABCA1 through NF-{kappa}B in macrophages and in phagocytes ingesting apoptotic cells. *Proc. Natl. Acad. Sci. USA.* **103:** 3112–3117.
14. Kaplan, R., X. Gan, J. G. Menke, S. D. Wright, and T. Q. Cai. 2002. Bacterial lipopolysaccharide induces expression of ABCA1 but not ABCG1 via an LXR-independent pathway. *J. Lipid Res.* **43:** 952–959.
15. Baranova, I., T. Vishnyakova, A. Bocharov, C. Zhigang, A. T. Remaley, J. Stonick, T. L. Eggerman, and A. P. Patterson. 2002. Lipopolysaccharide down regulates both scavenger receptor B1 and ATP binding cassette transporter A1 in RAW cells. *Infect. Immun.* **70:** 2995–3003.
16. Khovidhunkit, W., A. H. Moser, J. K. Shigenaga, C. Grunfeld, and K. R. Feingold. 2003. Endotoxin down-regulates ABCG5 and ABCG8 in mouse liver and ABCA1 and ABCG1 in J774 murine macrophages: differential role of LXR. *J. Lipid Res.* **44:** 1728–1736.
17. Castrillo, A., S. B. Joseph, S. A. Vaidya, M. Haberland, A. M. Fogelman, G. Cheng, and P. Tontonoz. 2003. Crosstalk between LXR and toll-like receptor signaling mediates bacterial and viral antagonism of cholesterol metabolism. *Mol. Cell.* **12:** 805–816.
18. Maitra, U., J. S. Parks, and L. Li. 2009. An innate immunity signaling process suppresses macrophage ABCA1 expression through IRAK-1-mediated downregulation of retinoic acid receptor {alpha} and NFATc2. *Mol. Cell. Biol.* **29:** 5989–5997.
19. Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch. 1990. Structure and function of lipopolysaccharide-binding protein. *Science.* **249:** 1429–1431.
20. Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science.* **249:** 1431–1433.
21. Haziot, A., E. Ferrero, F. Köntgen, N. Hijiya, S. Yamamoto, J. Silver, C. L. Stewart, and S. M. Goyert. 1997. Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity.* **4:** 407–414.
22. Poltorak, A., X. He, I. Smirnova, M-Y. H. C. Liu, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science.* **282:** 2085–2088.

23. Nagai, Y., S. Akashi, M. Nagafuku, M. Ogata, Y. Iwakura, S. Akira, T. Kitamura, A. Kosugi, M. Kimoto, and K. Miyake. 2002. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat. Immunol.* **3:** 667–672.
24. Foster, S. L., and R. Medzhitov. 2009. Gene-specific control of the TLR-induced inflammatory response. *Clin. Immunol.* **130:** 7–15.
25. Biswas, S. K., and E. Lopez-Collazo. 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends Immunol.* **30:** 475–487.
26. Foster, S. L., D. C. Hargreaves, and R. Medzhitov. 2007. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature.* **447:** 972–978.
27. Lu, M., M. Zhang, R. L. Kitchens, S. Fosmire, A. Takashima, and R. S. Munford. 2003. Stimulus-dependent deacylation of bacterial lipopolysaccharide by dendritic cells. *J. Exp. Med.* **197:** 1745–1754.
28. Lu, M., A. W. Varley, S. Ohta, J. Hardwick, and R. S. Munford. 2008. Host inactivation of bacterial lipopolysaccharide prevents prolonged tolerance following gram-negative bacterial infection. *Cell Host Microbe.* **4:** 293–302.
29. Lu, M., M. Zhang, A. Takashima, J. Weiss, M. A. Apicella, X. H. Li, D. Yuan, and R. S. Munford. 2005. Lipopolysaccharide deacylation by an endogenous lipase controls innate antibody responses to Gram-negative bacteria. *Nat. Immunol.* **6:** 989–994.
30. Shao, B., M. Lu, S. C. Katz, A. W. Varley, J. Hardwick, T. E. Rogers, N. Ojogun, D. C. Rockey, R. P. DeMatteo, and R. S. Munford. 2007. A host lipase detoxifies bacterial lipopolysaccharides in the liver and spleen. *J. Biol. Chem.* **282:** 13726–13735.
31. Kitchens, R. L., G. Wolfbauer, J. J. Albers, and R. S. Munford. 1999. Plasma lipoproteins promote the release of bacterial lipopolysaccharide from the monocyte cell surface. *J. Biol. Chem.* **274:** 34116–34122.
32. Kitchens, R. L., P. A. Thompson, S. Viriyakosol, G. E. O'Keefe, and R. S. Munford. 2001. Plasma CD14 decreases monocyte responses to LPS by transferring cell-bound LPS to plasma lipoproteins. *J. Clin. Invest.* **108:** 485–493.
33. Thompson, P. A., P. S. Tobias, S. Viriyakosol, T. N. Kirkland, and R. L. Kitchens. 2003. Lipopolysaccharide (LPS)-binding protein inhibits responses to cell-bound LPS. *J. Biol. Chem.* **278:** 28367–28371.
34. Basu, S. K., Y. K. Ho, M. S. Brown, D. W. Bilheimer, R. G. Anderson, and J. L. Goldstein. 1982. Biochemical and genetic studies of the apoprotein E secreted by mouse macrophages and human monocytes. *J. Biol. Chem.* **257:** 9788–9795.
35. Hashimoto, S., T. Suzuki, H-Y. Dong, N. Yamazaki, and K. Matsushima. 1999. Serial analysis of gene expression in human monocytes and macrophages. *Blood.* **94:** 837–844.
36. Mahley, R. W., and S. C. Jr. Rall. 2000. Apolipoprotein E: far more than a lipid transport protein. *Annu. Rev. Genomics Hum. Genet.* **1:** 507–537.
37. Zhang, W. Y., P. M. Gaynor, and H. S. Kruth. 1996. Apolipoprotein E produced by human monocyte-derived macrophages mediates cholesterol efflux that occurs in the absence of added cholesterol acceptors. *J. Biol. Chem.* **271:** 28641–28646.
38. Huang, Z. H., M. L. Fitzgerald, and T. Mazzone. 2006. Distinct cellular loci for the ABCA1-dependent and ABCA1-independent lipid efflux mediated by endogenous apolipoprotein E expression. *Arterioscler. Thromb. Vasc. Biol.* **26:** 157–162.
39. Rensen, P. C. N., M. Van Oosten, E. Van de Bilt, M. Van Eck, J. Kuiper, and T. J. C. Van Berkel. 1997. Human recombinant apolipoprotein E redirects lipopolysaccharide from Kupffer cells to liver parenchymal cells in rats in vivo. *J. Clin. Invest.* **99:** 2438–2445.
40. Fitzgerald, M. L., A. L. Morris, A. Chroni, A. J. Mendez, V. I. Zannis, and M. W. Freeman. 2004. ABCA1 and amphipathic apolipoproteins form high-affinity molecular complexes required for cholesterol efflux. *J. Lipid Res.* **45:** 287–294.
41. Vedhachalam, C., A. B. Ghering, W. S. Davidson, S. Lund-Katz, G. H. Rothblat, and M. C. Phillips. 2007. ABCA1-induced cell surface binding sites for ApoA-I. *Arterioscler. Thromb. Vasc. Biol.* **27:** 1603–1609.
42. Peet, D. J., S. D. Turley, W. Ma, B. A. Janowski, J. M. Lobaccaro, R. E. Hammer, and D. J. Mangelsdorf. 1998. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell.* **93:** 693–704.
43. Christiansen-Weber, T. A., J. R. Voland, Y. Wu, K. Ngo, B. L. Roland, S. Nguyen, P. A. Peterson, and W. P. Fung-Leung. 2000. Functional loss of ABCA1 in mice causes severe placental malformation, aberrant lipid distribution, and kidney glomerulonephritis as well as high-density lipoprotein cholesterol deficiency. *Am. J. Pathol.* **157:** 1017–1029.
44. Xie, C., S. D. Turley, and J. M. Dietschy. 2009. ABCA1 plays no role in the centripetal movement of cholesterol from peripheral tissues to the liver and intestine in the mouse. *J. Lipid Res.* **50:** 1316–1329.
45. Li, L., P. A. Thompson, and R. L. Kitchens. 2008. Infection induces a positive acute phase apolipoprotein E response from a negative acute phase gene: role of hepatic LDL receptors. *J. Lipid Res.* **49:** 1782–1793.
46. Munford, R. S., L. C. DeVeaux, J. E. Cronan, Jr., and P. D. Rick. 1992. Biosynthetic radiolabeling of bacterial lipopolysaccharide to high specific activity. *J. Immunol. Methods.* **148:** 115–120.
47. Kitchens, R. L., and R. S. Munford. 1998. CD14-dependent internalization of lipopolysaccharide (LPS) is strongly influenced by LPS aggregation but not by cellular responses to LPS. *J. Immunol.* **160:** 1920–1928.
48. Schultz, J. R., H. Tu, A. Luk, J. Repa, J. C. Medina, L. Li, S. Schwendner, S. Wang, M. Thoolen, D. J. Mangelsdorf, et al. 2000. Role of LXRs in control of lipogenesis. *Genes Dev.* **14:** 2831–2838.
49. Larrede, S., C. M. Quinn, W. Jessup, E. Frisdal, M. Olivier, V. Hsieh, M. J. Kim, M. van Eck, P. Couvert, A. Carrie, et al. 2009. Stimulation of cholesterol efflux by LXR agonists in cholesterol-loaded human macrophages is ABCA1-dependent but ABCG1-independent. *Arterioscler. Thromb. Vasc. Biol.* **29:** 1930–1936.
50. Yancey, P. G., H. Yu, M. F. Linton, and S. Fazio. 2007. A pathway-dependent on ApoE, ApoAI, and ABCA1 determines formation of buoyant high-density lipoprotein by macrophage foam cells. *Arterioscler. Thromb. Vasc. Biol.* **27:** 1123–1131.
51. Pradel, L. C., A. J. Mitchell, A. Zarubica, L. Dufort, L. Chansson, P. Naquet, C. Broccardo, and G. Chimini. 2009. ATP-binding cassette transporter hallmarks tissue macrophages and modulates cytokine-triggered polarization programs. *Eur. J. Immunol.* **39:** 2270–2280.
52. Gegner, J. A., R. J. Ulevitch, and P. S. Tobias. 1995. Lipopolysaccharide (LPS) signal transduction and clearance. Dual roles for LPS binding protein and membrane CD14. *J. Biol. Chem.* **270:** 5320–5326.
53. Chen, H., M. J. Cowan, J. D. Hasday, S. N. Vogel, and A. E. Medvedev. 2007. Tobacco smoking inhibits expression of proinflammatory cytokines and activation of IL-1R-associated kinase, p38, and NF-kappaB in alveolar macrophages stimulated with TLR2 and TLR4 agonists. *J. Immunol.* **179:** 6097–6106.
54. Von Baehr, V., W. D. Döcke, M. Plauth, C. Liebenthal, S. Küpferling, H. Lochs, R. Baumgarten, and H. D. Volk. 2000. Mechanisms of endotoxin tolerance in patients with alcoholic liver cirrhosis: role of interleukin 10, interleukin 1 receptor antagonist, and soluble tumour necrosis factor receptors as well as effector cell desensitisation. *Gut.* **47:** 281–287.
55. Brenchley, J. M., D. A. Price, T. W. Schacker, T. E. Asher, G. Silvestri, S. Rao, Z. Kazzaz, E. Bornstein, O. Lambotte, D. Altmann, et al. 2006. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat. Med.* **12:** 1365–1371.
56. Gao, L., Y. J. Tsai, D. N. Grigoryev, and K. C. Barnes. 2007. Host defense genes in asthma and sepsis and the role of the environment. *Curr. Opin. Allergy Clin. Immunol.* **7:** 459–467.