ABCA1 Protein Enhances Toll-like Receptor 4 (TLR4)-stimulated Interleukin-10 (IL-10) Secretion through Protein Kinase A (PKA) Activation

Loretta Ma†1, Fumin Dong†, Maryam Zaid‡, Ashok Kumar†, and Xiaohui Zha§2

From the †Ottawa Hospital Research Institute and ‡Department of Biochemistry, Microbiology and Immunology, University of Ottawa and the §Children’s Hospital of East Ontario, Ottawa, Ontario K1H 8L6, Canada

Background: ABCA1 is known to suppress proinflammatory cytokines. ABCA1 activates PKA and up-regulates anti-inflammatory cytokine IL-10. Elevated PKA transforms macrophages to M2-like phenotype. Disrupting lipid rafts by statins, MCD, and filipin recuperates ABCA1 phenotype and likely functions downstream of ABCA1.

Results: ABCA1 activates PKA and up-regulates anti-inflammatory cytokine IL-10. Elevated PKA transforms macrophages to M2-like phenotype. Disrupting lipid rafts by statins, MCD, and filipin recuperates ABCA1 phenotype and likely functions downstream of ABCA1.

Conclusion: By modulating cholesterol, ABCA1 activates PKA. This generates M2-like macrophages.

Significance: ABCA1 does not simply suppress inflammatory response. It promotes M2-like activation and facilitates resolution.

Nonresolving inflammatory response from macrophages is a major characteristic of atherosclerosis. Macrophage ABCA1 has been previously shown to suppress the secretion of proinflammatory cytokine. In the present study, we demonstrate that ABCA1 also promotes the secretion of IL-10, an anti-inflammatory cytokine critical for inflammation resolution. ABCA1+/+ bone marrow-derived macrophages secrete more IL-10 but less proinflammatory cytokines than ABCA1−/− bone marrow-derived macrophages, similar to alternatively activated (M2) macrophages. We present evidence that ABCA1 activates PKA and that this elevated PKA activity contributes to M2-like inflammatory response from ABCA1+/+ bone marrow-derived macrophages. Furthermore, cholesterol lowering by statins, methyl-β-cyclodextrin, or filipin also activates PKA and, consequently, transforms macrophages toward M2-like phenotype. Conversely, cholesterol enrichment suppresses PKA activity and promotes M1-like inflammatory response. As the primary function of ABCA1 is cholesterol removal, our results suggest that ABCA1 activates PKA by regulating cholesterol. Indeed, forced cholesterol enrichment in ABCA1-expressing macrophages suppresses PKA activation and elicits M1-like response. Collectively, these findings reveal a novel protective process by ABCA1-activated PKA in macrophages. They also suggest cholesterol lowering in extra-hepatic tissues by statins as an anti-inflammatory strategy.

The onset of atherosclerosis is characterized by two fundamental hallmarks: cholesterol accumulation and inflammation, particularly of macrophages. Cholesterol accumulation is due to elevated plasma cholesterol and, consequently, building up of cholesterol-rich lipoproteins within the artery, resulting in recruitment and retention of macrophages. Macrophages are subsequently converted into cholesterol-loaded foam cells by engulfing lipoproteins. Foam cells further fuel inflammation by secreting proinflammatory cytokines, thereby delaying and impairing inflammation resolution. Although the detailed mechanisms for this action remain largely elusive, excess cholesterol in macrophages is thought to hyperactivate inflammatory response such as those triggered by Toll-like receptors (TLRs)3 (2). Indeed, cholesterol accumulation was shown to exacerbate LPS-stimulated secretion of TNF-α and other proinflammatory cytokines (3).

Excess cholesterol in macrophages is normally countered by cholesterol efflux mediated by ABC transporters. ABCA1 primarily facilitates cholesterol and phospholipid efflux to lipoprotein-poor apolipoprotein A-I (apoA-I), which generates nascent HDL (4, 5). Individuals defective in ABCA1 have almost no HDL and elevated atherosclerosis. Another ABC transporter, ABCG1, also removes cholesterol but has been suggested to function downstream of ABCA1 as it needs nascent HDL as acceptor (6). Interestingly, both ABCA1 and ABCG1 have significant impact on inflammation. Macrophages without ABCA1 or ABCG1 secrete more proinflammatory cytokines even in the absence of excessive cholesterol loading (3, 7). ABCA1/ABCG1 double deletion further exacerbates this response (7). Currently, this is thought to be primarily due to increased recruitment of TLRs to the cholesterol-rich membrane microdomains such as lipid rafts, which is more abundant in the absence of ABCA1 or ABCG1 (8). However, the precise mechanisms by which these ABC transporters or cholesterol modulate TLR-mediated immune response are largely unknown.

It is widely accepted now that atherosclerosis is a chronic inflammatory disease, marked by dysregulation of the inflam-
ABCA1 Activates PKA to Secrete IL-10

ABCA1 Expression Enhances IL-10 Secretion—It has been widely reported that ABCA1 suppresses TLR4-mediated TNF-α secretion in various tissue culture and animal models. To test whether ABCA1 enhances the release of anti-inflammatory cytokines at the same time, BMDM from WT and ABCA1<sup>−/−</sup> mice were stimulated with LPS, a TLR4 ligand. The medium was first analyzed using a cytokine array (supplemen-

RESULTS

ABCA1 Activates PKA to Secrete IL-10

ABCA1 expression by binding liver X receptor agonist, increases ABCA1 gene expression by binding liver X receptor response element within the ABCA1 gene promoter (17). Mouse BMDM were obtained by flushing the femurs of ABCA1<sup>−/−</sup> and ABCA1<sup>+/+</sup> C57 mice and allowed to differentiate into macrophages by incubation with DMEM containing 10% FBS and 10% L929 conditioned medium for 7 days.

Cytokine ELISA Assay—RAW264.7 and BMDM were first induced to express ABCA1 with 10 μM T0901317 for 18–20 h. Some of the cells were pretreated with either 50 μM PKI or other compounds, such as methyl-β-cyclodextrin (MCD) or filipin, and then stimulated with 100 ng/ml LPS in DMEM supplemented with 1 mg/ml BSA for 6 h. The medium was collected, and cell debris was removed by centrifuging at 12,000 × g for 5 min. Cells were lysed with 1× SDS lysis buffer (50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol, protease inhibitor mixture, and one tablet of PhosSTOP per 10 ml of buffer). IL-10 and TNF-α in the medium were determined using a kit from R&D Systems Inc. Protein levels in cell lysates were used to normalize cytokine levels in the medium.

Cytokine Array Assay—BMDM cells were induced to express ABCA1 with 10 μM T0901317 for 18–20 h. Cells were incubated with 100 ng/ml LPS in DMEM supplemented with 1 mg/ml BSA for 6 h. Medium and cells were collected as described above. Cytokine in the medium was determined using RayBio® Cytokine Antibody Array 1.

Immunofluorescent Staining—BHK cells were seeded in glass bottom coverslip microscopy dishes and grown to 50–70% confluency. Cells were fixed with 4% paraformaldehyde in PBS for 10 min and subsequently permeabilized with 0.1 mg/ml saponin in PBS for 30 min. Nonspecific binding was blocked with 5% calf serum and 50 mM NH<sub>4</sub>Cl in PBS for 20 min. The primary antibodies (P-PKA substrate or ABCA1) were then added at 1:200 in 5% calf serum/PBS for 30 min followed by incubation with secondary antibodies (Alexa Fluor-488 goat anti-rabbit IgG and Alexa Fluor-547 goat anti-mouse IgG, 1:200) for 30 min. Fluorescent images were taken using a Nikon TE2000-E inverted fluorescent microscope with a 60× objective. Identical settings were used to take images of ABCA1 and mock cells. Fluorescent intensities of individual cells were analyzed with MetaMorph software.

Statistics—Statistical analyses between data groups were performed with PRISM software (GraphPad). Data for Western blot analyses and ELISA experiments are presented as the mean ± S.E. or S.D. as indicated. For quantification of immunoblots, relative unit values were measured using the Image Lab software. The statistical significance of differences between groups was analyzed by Student’s t test. Differences were considered significant at a p value < 0.05.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Cell culture growth medium, antibiotics (penicillin and streptomycin), and fetal calf serum (FCS) were purchased from Invitrogen. Baby hamster kidney (BHK) cells that are stably transfected with a mifepristone-inducible vector with or without ABCA1 gene insert were from Drs. Oram and Vaughan (University of Washington, Seattle, WA). The RAW 264.7 cell line was purchased from the ATCC. Mouse bone marrow-derived macrophages were kindly provided by Dr. Marcel (Ottawa University Heart Institute). Mifepristone was from Invitrogen, and T0901317 was from Sigma. The following antibodies were acquired from the following vendors: mouse monoclonal anti-ABCA1 (Upstate Millipore), rabbit polyclonal anti-phosphorylated-PKA substrate (Cell Signaling), rabbit polyclonal anti-phosphorylated-CREB (Cell Signaling), mouse monoclonal anti-CREB (Cell Signaling), and mouse monoclonal anti-Hsp70 (BD Transduction Laboratories). Protease inhibitor mixture and phosphatase inhibitor (PhosSTOP) were purchased from Roche Applied Science. Methyl-β-cyclodextrin, filipin, compactin, simvastatin, and mevalonate were from Sigma-Aldrich, and PKA inhibitor (PKI) was from Enzo Bioscience.

Cell Cultures—Both BHK cells and RAW 264.7 macrophage cells were maintained in DMEM supplemented with 10% FCS at 37 °C in a 5% CO<sub>2</sub> incubator. ABCA1 expression was induced during 16–18 h of incubation in DMEM with 1 mg/ml BSA containing either 5 nm mifepristone or 10 μM T0901317 for BHK or RAW264.7 and BMDM, respectively. Mock-transfected cells were used as negative controls in experiments with BHK cells, whereas T0901317 was withheld for negative con-
trols in experiments with RAW cells. T0901317, a liver X receptor agonist, increases ABCA1 gene expression by binding liver X receptor response element within the ABCA1 gene promoter (17). Mouse BMDM were obtained by flushing the femurs of ABCA1<sup>−/−</sup> and ABCA1<sup>+/+</sup> C57 mice and allowed to differentiate into macrophages by incubation with DMEM containing 10% FBS and 10% L929 conditioned medium for 7 days.
Consistent with previous findings including ours (3, 18), ABCA1/H11001/H11001 BMDM (WT) secreted fewer proinflammatory cytokines, such as TNF-α and IL-12p40 (Fig. 1A), in comparison with ABCA1/H11002/H11002 BMDM. However, ABCA1 expression in BMDM also significantly enhanced the secretion of IL-10. This enhanced IL-10 secretion was further verified by ELISA in both primary BMDM and RAW macrophages; ABCA1-expressing macrophages produced significantly more IL-10 but less TNF-α (Fig. 1, B and C). Collectively, these results reveal an important and novel immune regulatory function of ABCA1, not simply immune suppression as previously reported (3).

**ABCA1 Activates PKA, and This Activation Requires Functional ATP Binding Domain of ABCA1**—ABCA1 has been reported to decrease TLR4 surface presentation and recruitment to lipid rafts (7, 19), which could explain less TNF-α release by LPS. However, as we showed above, ABCA1 also robustly enhances IL-10 secretion. As LPS/TLR4 is required for the release of both IL-10 and TNF-α (neither was detectable without LPS), the initial TLR4 signaling, i.e. TLR4 surface presentation or recruitment to lipid raft, is not likely compromised significantly by ABCA1. Rather, some factors, which are induced by ABCA1 and act downstream of the initial TLR4 signaling, have poised macrophages toward an M2-like response. One candidate of such factors is PKA. PKA activation is known to switch macrophages to M2-like responses, i.e. high IL-10 and low TNF-α secretion (16). We thus wondered whether ABCA1 could activate PKA. To test this, we first analyzed the phosphorylation of CREB, a PKA substrate, in BHK cells. These cells are stable transfectants that inducibly express ABCA1, its mutants, and mock cells (generated with identical plasmids but without ABCA1 insert), respectively (20). We found that p-CREB level is clearly elevated in BHK cells.

**FIGURE 1. ABCA1 expression in primary mouse BMDM increases IL-10 secretion and decreases proinflammatory cytokine secretion.** WT and ABCA1−/− primary mouse BMDM were induced with 10 μM T0901317 overnight followed by 100 ng/ml LPS treatment for 6 h. A, A mouse cytokine array (see supplemental data) was used to determine the levels of cytokine secretion from WT and ABCA1−/− BMDM. C and B, IL-10 and TNFα levels in the medium from primary mouse BMDM (B) and RAW 264.7 macrophages (C) were measured by mouse cytokine ELISAs. Data are presented as average of duplicated samples with S.D.
expressing WT ABCA1, relative to that of mock cells (Fig. 2A).
A nonfunctional ABCA1 mutant, A937V, failed to increase p-CREB despite being expressed at a similar level as WT ABCA1 (Fig. 2A) with correct targeting to the plasma membrane (21). ABCA1<sup>A937V</sup> is defective in ATP binding and consequently unable to efflux cholesterol to apoA-I or perturb lipid rafts in the plasma membrane (20, 21). This suggests that PKA activation is a functional consequence of ABCA1. Indeed, such PKA activation by ABCA1 was further confirmed in macrophages; WT BMDM exhibited higher levels of p-CREB when compared with ABCA1<sup>−/−</sup> BMDM (Fig. 2B), which was also observed with ABCA1-expressing RAW macrophages (Fig. 2C).

To further substantiate these observations, we next assessed PKA activity with an antibody raised against PKA-phosphorylated proteins. Consistent with p-CREB results above, there were more PKA-phosphorylated proteins in ABCA1-expressing BHK cells relative to those in mock cells (Fig. 3A). PKA-phosphorylated proteins were also significantly elevated in ABCA1<sup>+/+</sup> BMDM in comparison with ABCA1<sup>−/−</sup> BMDM (Fig. 3B). Similarly, RAW macrophages had more PKA-phosphorylated proteins when induced to express ABCA1 (Fig. 3C). Furthermore, elevation of PKA-phosphorylated proteins by ABCA1 was clearly observed at the single BHK cell level by microscopy. Higher ABCA1 expression is correlated with more PKA-phosphorylated proteins (Fig. 3D, arrows). Conversely, adjacent cells with low or little ABCA1 expression have fewer PKA-phosphorylated proteins (Fig. 3D, arrowheads), similar to that of the mock cells. A positive correlation between ABCA1 expression level and the level of PKA-phosphorylated proteins was also observed from a large number of individual cells (Fig. 3E). Together with p-CREB results, we conclude that there is most likely a causal relationship between ABCA1 function and PKA activity.

**PKA Activity Is Required for ABCA1 to Exert Its Immune Regulatory Function**—We next investigated whether elevated PKA activity by ABCA1 was at least partially responsible for the more favorable IL-10/TNF-α release profile. For this, we used a cell-permeable short peptide (6 amino acids), PKI, to acutely block PKA function. One technical limitation is that ABCA1<sup>+/+</sup> BMDM had been expressing ABCA1 for more than 24 h since induction. Accordingly, PKA activity should be continuously elevated during this period. To avoid a broad effect, we only pretreated BMDM with PKI for 30 min before LPS addition. Such short treatment may not be able to completely reverse all the proteins phosphorylated by ABCA1-activated PKA, particularly for those with a slow dephosphorylation rate. Nevertheless, as shown in Fig. 4A, PKI at a concentration with negligible effect on ABCA1<sup>−/−</sup> BMDM, was able to significantly suppress IL-10 secretion from ABCA1<sup>+/+</sup> BMDM. Similarly, ABCA1<sup>+/+</sup> BMDM released more TNF-α in the presence of PKI when ABCA1<sup>−/−</sup> BMDM was not significantly altered (Fig. 4B). These results demonstrate that PKA activation is significantly contributing to the regulation of inflammatory response by ABCA1.

**Cholesterol Influences Steady State PKA Activity**—To understand how ABCA1 activates PKA, we reviewed some well-established ABCA1 functions. The first and foremost, ABCA1 interacts with apoA-I and mediates cholesterol efflux to apoA-I. ApoA-I is known to increase PKA activity (22) and suppress TNF-α secretion (9, 18). However, all the cell models used here including macrophages were not at all exposed to apoA-I; they were induced to express ABCA1 in BSA-only medium. ApoA-I therefore cannot be a significant contributor to PKA activation observed here. Another widely reported observation is that ABCA1 decreases lipid raft content in the plasma membrane (18, 19, 21), independent of apoA-I. Thus, we tested whether
modulating lipid rafts influences PKA activity in macrophages. This was first achieved by incubating RAW macrophages with increasing concentrations of MCD for 30 min. MCD is a cholesterol-sequestering agent, thereby removing cholesterol from the plasma membrane (23). This in general depletes lipid rafts (24). We found that p-CREB levels were dose-dependently elevated by MCD (Fig. 5A). Also, macrophages were treated with filipin for 1 h. Filipin is known to bind free cholesterol on the plasma membrane. This sequesters cholesterol away from the general area of the plasma membrane. We observed a similar increase of p-CREB in filipin-treated cells (Fig. 5B). Furthermore, macrophages were treated with statins in lipoprotein-

FIGURE 3. PKA phosphorylation is higher with ABCA1-expressing cells. Cells were prepared as in Fig. 2. A–C, cell lysates were immunoblotted (IB) for phospho-PKA substrates of BHK cells (A), BMDM (B), and RAW macrophages (C). D, immunofluorescence staining of BHK cells with anti-ABCA1 and antiphosphorylated PKA substrate antibodies (anti-P-PKA-S). Na/K-ATPase, Parp-1, and Hsp70 were used as loading control, respectively. E, fluorescent intensities per cell (FI/cell) as a correlation between the level of phospho-PKA substrates and level of ABCA1 expression in individual cells.
ABCA1 Activates PKA to Secrete IL-10

The major conclusion from this study is that ABCA1 activates PKA. Elevated PKA activity significantly assists ABCA1 to poise macrophages to an M2-like response when exposed to LPS, such as releasing more IL-10 but less TNF-α. IL-10 is a major anti-inflammatory cytokine. To our knowledge, this is the first study demonstrating the role of ABCA1 in enhancing the anti-inflammatory arm of the immune response.

It is generally accepted that atherosclerosis is initiated and propagated by the response of the innate immune system. The innate immune system has evolved to mount robust response to infection and injury and, at the same time, be self-limiting to avoid excessive damage and to promote recovery. As such, a well orchestrated balance of pro- and anti-inflammatory programs is essential for the eventual inflammation resolution. Atherosclerosis is a chronic inflammatory disease and, in a sense, the consequence of excessive proinflammatory activities and failure to proceed to inflammation resolution. Indeed, in this context, strengthening the anti-inflammatory arm has proven to be beneficial. For example, IL-10-deficient mice develop more fatty streaks when compared with WT animals when fed high fat diet (25). By contrast, IL-10 transgenic mice do not develop fatty streaks under the same condition (26). Also, IL-10 deficiency in apoE^{-/-} mice increases atherosclerosis (27). ABCA1 is significantly antiatherogenic both in human and in all animal models tested to date. As we reported here, ABCA1 enhances IL-10 secretion but limits TNF-α release, in addition to its role in HDL biogenesis. It is thus tempting to speculate that ABCA1 may prevent atherosclerosis partially by promoting M2-like immune responses.

It is known that macrophages can undergo classical (M1) or alternative (M2) activation, which represent extremes of a con-
ABCA1 Activates PKA to Secrete IL-10

continuum in a universe of activation states (28). Among other things, the M1 phenotype is characterized by the expression of high levels of proinflammatory cytokines/chemokines. In contrast, M2 macrophages have immune regulatory functions, characterized by efficient phagocytic activity, high expression of scavenging molecules, and an IL-12lowIL-10hi phenotype. Although we only analyzed IL-10 in the current study, Chimini and colleagues (29) have performed a comprehensive screen of ABCA1-expressing or -nonexpressing macrophages from various mouse models and concluded that ABCA1 is a positive factor to promote the appearance of M2 markers, including CD163 and ARG1. In fact, ABCA1 itself behaves as an exquisite M2 marker as its expression is positively regulated by IL-4 and parallels the expression of most established M2 markers (29), This is in good agreement with our observations here. Also, M2 macrophages exhibit enhanced IL-10 but suppressed TNF-α secretion when challenged by LPS. Interestingly, IL-10 itself can also significantly promote M1 to M2 transition (30). In this context, by promoting IL-10 secretion, ABCA1 should further strengthen and enrich M2 phenotypes, thereby facilitating inflammation resolution.

We provide evidence that this immune regulatory function of ABCA1 is at least partially due to its ability to activate PKA. It has long been established that cAMP elicits an anti-inflammatory effect on the innate immune system (14). cAMP can activate both PKA and exchange proteins activated by cAMP (Epac). However, its immune regulatory function was recently reported to be primarily through PKA activation in macrophages. For example, PKA knockdown completely abolishes the immune regulatory effects of cAMP, whereas Epac antagonist has little effect (16). At the molecular level, cAMP activates PKA to phosphorylate several immune regulatory molecules including NF-κB p65 and CREB. This leads to the suppression of NF-κB-mediated proinflammatory cytokine expression but

FIGURE 5. Cholesterol depletion increases p-CREB. A–C, RAW macrophages were treated with 0, 1, or 5 mM MCD for 30 min (A), 3 μg/ml filipin for 1 h (B), or 3, 5, or 7 μM statins (compactin and simvastatin) for 48 h (C). Cell lysates were immunoblotted for p-CREB and total CREB with Hsp70 as loading control. Bar graphs represent averages from at least two independent experiments with standard deviations. LPDS, lipoprotein-deficient serum.
enhancement of IL-10 generation, respectively (16). Interestingly, ABCA1 can also activate STAT3 through apoA-I, which is known to up-regulate IL-10 expression. However, without apoA-I as in the present study, ABCA1 is unable to influence STAT3 activity (9). Therefore, STAT3 is not likely the direct mechanism by which ABCA1 promotes IL-10 secretion.

The mechanisms by which ABCA1 increases PKA activity remain to be elucidated. Elevated cellular cAMP can certainly increase PKA activity. Alternatively, PKA activity could be regulated by PKA cellular localizations. In recent years, it has become increasingly recognized that intracellular cAMP is distributed in a highly non-uniform fashion. For example, many PKA-anchoring proteins, i.e. AKAPs, also anchor adenylyl cyclases to produce cAMP and diesterases to degrade cAMP locally. This provides a localized and also temporal pool of cAMP for PKA activation. ABCA1 can increase PKA activity potentially by influencing any of these molecules.

However, the most well established function of ABCA1 is its regulation of cholesterol. ABCA1 regulates cellular cholesterol at two levels. First, ABCA1 facilitates cholesterol efflux to apoA-I. This decreases overall cellular cholesterol contents. Secondly, ABCA1 weakens cholesterol interaction with phospholipids in the membrane, perhaps similar to flippases, and disrupts the formation of microdomains, such as lipid rafts. These cholesterol regulation functions are likely essential for PKA activation by ABCA1. Indeed, once cholesterol-loaded, ABCA1 fails to elevate PKA and also fails to poise macrophage for the M2-like inflammatory responses. These cholesterol-enriched macrophages secrete more TNF-α but less IL-10 than non-loaded cells, although ABCA1 expression remains unchanged. Thus, it is likely that cholesterol acts more proximally than ABCA1 to PKA activation. Consistent with this notion, we found that cholesterol depletion by various reagents, a common approach for lipid raft disruption, activates PKA and modulates cytokine secretion accordingly, without change in ABCA1 expression.

Interestingly, similar cholesterol manipulations were found to increase adenylyl cyclase activity. For example, in

**FIGURE 6. Cholesterol depletion increases PKA-phosphorylated proteins.** A and B, RAW macrophages were pretreated with or without 50 μM PKI for 1 h and subsequently treated 30 min with 5 mM MCD or 10 μg/ml filipin (A) or incubated with statins in 10% lipoprotein-deficient serum (LPDS) medium for 48 h (B). Whole cell lysates were analyzed by immunoblotting for phospho-PKA substrates with Hsp70 as loading control. Bar graphs represent averages from multiple independent experiments with standard deviations.

**FIGURE 7. Cholesterol depletion promotes IL-10 secretion but suppresses TNF-α release.** A, RAW macrophages were treated with 10 μM T0901317 overnight and followed by 100 ng/ml LPS treatment in combination with MCD or filipin for 6 h. B, some of the cells were preincubated with 50 μM PKI and then treated as above. Medium IL-10 and TNF-α levels were measured by ELISA. Data are presented as average of duplicated samples with S.D.
cells treated with MCD (and therefore with fewer lipid rafts), β₂ adrenergic receptor can more efficiently form a complex with adenylyl cyclase and G protein (Gᵢ). This leads to activation of adenylyl cyclase and increased cAMP production both under basal condition (i.e. without β₂ adrenergic receptor stimuli) and with stimulation (32). PKA could be activated in ABCA1-expressing cells by similar adenylyl cyclase-mediated mechanism. This higher steady state PKA activity then poises macrophages to M2-like inflammatory responses.

It has been suggested that lowering cellular cholesterol (thus resulting in fewer lipid rafts) disrupts initial TLR4 signaling, resulting in an overall suppression of inflammatory response. Indeed, high concentration of MCD is known to inhibit MyD88-dependent TLR recruitment to lipid rafts and thus prevent TLR from forming complexes with accessory proteins (33).
Drawing an analogy to this, ABCA1 is speculated to suppress TLRs, particularly TLR4, by removing cholesterol and disrupting lipid rafts. ABCA1 is widely reported to suppress LPS-stimulated TNF-α release. However, the secretion of IL-10, another direct downstream event of LPS-TLR4 signaling, is increased by ABCA1 as we reported here. This argues against a simple immune suppression. Rather, by activating PKA, ABCA1 poises macrophages to a different state. Such a state allows macrophages to mount a distinct and more favorable response when challenged by LPS. Intriguingly, a recent comprehensive genome-wide analysis in macrophages suggests that LPS-stimulated gene expression primarily results from LPS-independent transcription factor positioning (therefore poised). LPS stimulation merely amplifies the transcription from this predetermined positioning pattern (34). It would be interesting to see whether ABCA1 or cholesterol depletion influences this prepositioning of transcription factors. Perhaps PKA could influence this event. On this note, PKA was shown to modulate TLR4 inflammatory responses through a nuclear PKA-anchoring protein, AKAP-95 (16). AKAP-95 was recently reported to regulate the activity of S6 kinase (S6k), a key mediator of mTORC1 to regulate mRNA transcription (31). Also consistent with this global prepositioning (or a poised state), the work from Chimini and colleagues (29) concluded that ABCA1+/- macrophages are M2-like, versus an M1-like phenotype in ABCA1-/- macrophages without inflammatory stimuli.

Another relevant and perhaps equally important finding here is how cholesterol loading with modified LDL tips the balance toward M1-like immune response in macrophages. Conversely, cholesterol lowering by statins switches macrophages toward M2-like inflammatory response. Given that the primary cause of atherosclerosis is the elevated LDL in the circulation, it is plausible that high cholesterol weakens the defense capacity of the immune system by suppressing macrophage M2 polarization. In support of this, statins are known to offer anti-inflammatory functions, independent of their LDL-lowering capacity. Perhaps statins could also decrease cellular cholesterol in peripheral tissues including macrophages, in addition to its well-established up-regulation of LDL receptor in the liver. This could make immune cells more resilient to environmental challenges, thereby resulting in less chronic inflammation and less atherosclerosis.

In summary, the present study demonstrates for the first time that ABCA1 directly promotes the anti-inflammatory arm of the immune response. This is most likely through PKA activation. Perhaps equally importantly, we provide evidence that cholesterol has a direct role in immune regulation.

Acknowledgment—We thank Dr. Yves Marcel for critically reading manuscript and providing bone marrow-derived macrophages.

REFERENCES

1. Williams, K. J., and Tabas, I. (1995) The response-to-retention hypothesis of early atherogenesis. Arterioscler. Thromb. Vasc. Biol. 15, 551–561
2. Sun, Y., Ishibashi, M., Seimon, T., Lee, M., Sharma, S. M., Fitzgerald, K. A., Samokhin, A. O., Wang, Y., Sayers, S., Aikawa, M., Jerome, W. G., Ostrowski, M. C., Bromme, D., Libby, P., Tabas, I. A., Welch, C. L., and Tall, A. R. (2009) Free cholesterol accumulation in macrophage membranes activates Toll-like receptors and p38 mitogen-activated protein kinase and induces cathespin K. Circ. Res. 104, 455–465
3. Zhu, X., Lee, J. Y., Timmins, J. M., Brown, J. M., Boudygina, E., Mulya, A., Gebre, A. K., Willingham, M. C., Hiltbold, E. M., Mishra, N., Maeda, N., and Parks, J. S. (2008) Increased cellular free cholesterol in macrophage-specific Aβca1 knock-out mice enhances pro-inflammatory response of macrophages. J. Biol. Chem. 283, 22930–22941
4. Oram, J. F., and Lawn, R. M. (2001) ABCA1: the gatekeeper for eliminating excess tissue cholesterol. J. Lipid Res. 42, 1173–1179
5. Attie, A. D., Kastelein, J. P., and Hayden, M. R. (2001) Pivotal role of ABCA1 in reverse cholesterol transport influencing HDL levels and susceptibility to atherosclerosis. J. Lipid Res. 42, 1717–1726
6. Vaughan, A. M., and Oram, J. F. (2006) ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL. J. Lipid Res. 47, 2433–2443
7. Yvan-Charvet, L., Welch, C., Pagler, T. A., Ranalletta, M., Lamkanfi, M., Han, S., Ishibashi, M., Li, R., Wang, N., and Tall, A. R. (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
8. Yvan-Charvet, L., Wang, N., and Tall, A. R. (2010) Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses. Arterioscler. Thromb. Vasc. Biol. 30, 139–143
9. Tang, C., Liu, Y., Kessler, P. S., Vaughan, A. M., and Oram, J. F. (2009) The macrophage cholesterol exporter ABCA1 functions as an anti-inflammatory receptor. J. Biol. Chem. 284, 32336–32343
10. Alvarez, Y., Municic, C., Alonso, S., Sánchez Crespo, M., and Fernández, N. (2009) The induction of IL-10 by zymosan in dendritic cells depends on CREB activation by the coactivators CREB-binding protein and TORC2 and autocrine PGE2. J. Immunol. 183, 1471–1479
11. Mellett, M., Atzei, P., Jackson, R., O’Neill, L. A., and Moynagh, P. N. (2011) Mal mediates TLR-induced activation of CREB and expression of IL-10. J. Immunol. 186, 4925–4935
12. Natarajan, M., Lin, K. M., Hushe, R. C., Sternweis, P. C., and Ranganathan, R. (2006) A global analysis of cross-talk in a mammalian cellular signalling network. Nat. Cell Biol. 8, 571–580
13. Pradervand, S., Maurya, M. R., and Subramaniam, S. (2006) Identification of signaling components required for the prediction of cytokine release in RAW 264.7 macrophages. Genome Biol. 7, R11
14. Bourne, H. R., Lichtenstein, L. M., Melmon, K. L., Henney, C. S., Weinstein, Y., and Shearer, G. M. (1974) Modulation of inflammation and immunity by cyclic AMP. Science 184, 19–28
15. Lawrence, T., and Natoli, G. (2011) Transcriptional regulation of macrophage polarization: enabling diversity with identity. Nat. Rev. Immunol. 11, 750–761
16. Wall, E. A., Zavzavadjian, J. R., Chang, M. S., Randhawa, B., Zhu, X., Hushe, R. C., Liu, J., Driver, A., Bao, X. R., Sternweis, P. C., Simon, M. I., and Fraser, I. D. (2009) Suppression of LPS-induced TNF-α production in macrophages by cAMP is mediated by PKA-AKAP95-p105. Sci. Signal. 2, ra28
17. Schwartz, K., Lawn, R. M., and Wade, D. P. (2000) ABC1 gene expression and ApoA-I-mediated cholesterol efflux are regulated by LXR. Biochem. Biophys. Res. Commun. 274, 794–802
18. Ma, L., Dong, F., Denis, M., Feng, Y., Wang, M. D., and Zha, X. (2011) HI31, a protein kinase A anchoring inhibitor, induces robust cholesterol efflux and reverses macrophage foam cell formation through ATP-binding cassette transporter A1. J. Biol. Chem. 286, 3370–3378
19. Zhu, X., Owen, J. S., Wilson, M. D., Li, H., Griffiths, G. L., Thomas, M. I., Hiltbold, E. M., Fessler, M. B., and Parks, J. S. (2010) Macrophage ABCA1 reduces MyD88-dependent Toll-like receptor trafficking to lipid rafts by reduction of lipid raft cholesterol. J. Lipid Res. 51, 3196–3206
20. Vaughan, A. M., and Oram, J. F. (2003) ABCA1 redistributes membrane cholesterol independent of apolipoprotein interactions. J. Lipid Res. 44, 1373–1380
21. Landry, Y. D., Denis, M., Nandi, S., Bell, S., Vaughan, A. M., and Zha, X. (2006) ATP-binding cassette transporter A1 expression disrupts raft membrane microdomains through its ATPase-related functions. J. Biol.
22. Haidar, B., Denis, M., Marcil, M., Krimbou, L., and Genest, J., Jr. (2004) Apolipoprotein A-I activates cellular cAMP signaling through the ABCA1 transporter. *J. Biol. Chem.* **279**, 9963–9969

23. Pitha, J., Irie, T., Sklar, P. B., and Nye, J. S. (1988) Drug solubilizers to aid pharmacologists: amorphous cyclodextrin derivatives. *Life Sci.* **43**, 493–502

24. Simons, K., and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31–39

25. Mallat, Z., Besnard, S., Duriez, M., Deleuze, V., Emmanuel, F., Bureau, M. F., Soubrier, F., Esposito, B., Duez, H., Fievet, C., Staels, B., Duverger, N., Scherman, D., and Tedgui, A. (1999) Protective role of interleukin-10 in atherosclerosis. *Circ. Res.* **85**, e17–e24

26. Pinderski Oslund, L. J., Hedrick, C. C., Olvera, T., Hagenbaugh, A., Territo, M., Berliner, J. A., and Fyfe, A. I. (1999) Interleukin-10 blocks atherosclerotic events *in vitro* and *in vivo*. *Arterioscler. Thromb. Vasc. Biol.* **19**, 2847–2853

27. Caligiuri, G., Rudling, M., Ollivier, V., Jacob, M. P., Michel, J. B., Hansson, G. K., and Nicoletti, A. (2003) Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. *Mol. Med.* **9**, 10–17

28. Sica, A., and Mantovani, A. (2012) Macrophage plasticity and polarization: *in vivo* veritas. *J. Clin. Invest.* **122**, 787–795

29. Pradel, L. C., Mitchell, A. J., Zarubica, A., Dufort, L., Chasson, L., Naquet, P., Broccardo, C., and Chimini, G. (2009) ATP-binding cassette transporter hallmarks tissue macrophages and modulates cytokine-triggered polarization programs. *Eur. J. Immunol.* **39**, 2270–2280

30. Deng, B., Wehling-Henricks, M., Villalta, S. A., Wang, Y., and Tidball, J. G. (2012) IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. *J. Immunol.* **189**, 3669–3680

31. Gao, X., Chaturvedi, D., and Patel, T. B. (2012) Localization and retention of p90 ribosomal S6 kinase 1 in the nucleus: implications for its function. *Mol. Biol. Cell* **23**, 503–515

32. Pontier, S. M., Percherancier, Y., Galandrin, S., Breit, A., Galés, C., and Bouvier, M. (2008) Cholesterol-dependent separation of the β2-adrenergic receptor from its partners determines signaling efficacy: insight into nanoscale organization of signal transduction. *J. Biol. Chem.* **283**, 24659–24672

33. Triantafilou, M., and Triantafilou, K. (2002) Lipopolysaccharide recognition: CD14, TLRs, and the LPS-activation cluster. *Trends Immunol.* **23**, 301–304

34. Escoubet-Lozach, L., Benner, C., Kaikkonen, M. U., Lozach, J., Heinz, S., Spann, N. J., Crotti, A., Stender, J., Ghisletti, S., Reichart, D., Cheng, C. S., Luna, R., Ludka, C., Sasik, R., Garcia-Bassets, I., Hoffmann, A., Subramaniam, S., Hardiman, G., Rosenfeld, M. G., and Glass, C. K. (2011) Mechanisms establishing TLR4-responsive activation states of inflammatory response genes. *PLoS Genet.* **7**, e1002401