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Adenylyl Cyclase 6 Activation Negatively Regulates TLR4 Signaling through Lipid Raft–Mediated Endocytosis

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Proper intracellular localization of TLRs is essential for their signaling and biological function. Endocytosis constitutes a key step in protein turnover, as well as maintenance of TLR localization in plasma membrane and intracellular compartments, and thus provides important regulating points to their signaling. In this study, we demonstrate that adenylyl cyclase (AC) activation attenuates TLR4 signaling in a murine macrophage cell line (RAW 264.7) and bone marrow–derived macrophages when stimulated with LPS. We further show that the AC6 isoform plays a key role in negative regulation of TLR4 signaling by promoting protein degradation. TLR4 is normally endocytosed through the clathrin-mediated pathway, but concomitant AC6 activation shifts it to lipid raft-mediated endocytosis, which accelerates degradation of TLR4 and suppresses downstream signaling. Our studies unveil a new mechanism of negative regulation of TLR4 signaling through AC6-mediated endocytosis, which might provide a novel therapeutic approach for limiting inflammatory and autoimmune diseases. ***The Journal of Immunology*****, 2013, 191: 6093–6100.**

Eukaryotic cells use endocytosis for internalization of nutrients, regulation of signal transduction, elimination of pathogens, presentation of Ags, and an array of other physiological processes (1–4). Besides phagocytosis, endocytosis can be further categorized into three broad pathways: micropinocytosis, clathrin-mediated endocytosis (CME), and clathrin-independent endocytosis (5, 6). Many efforts have been focused on CME (7, 2), whereas clathrin-independent endocytosis is still poorly understood (7, 8). One subset of the clathrin-independent pathway involves caveolin/lipid rafts, which have attracted much recent attention due to its widespread cross talk with various signaling pathways and involvement in diverse biological events such as lymphocyte activation (9, 10). Generally, membrane proteins are internalized through one major pathway, and in some cases, blocking the pathway can activate alternative pathways. Some receptors, including the TGF-β receptor, use different internalization pathways for distinct purposes. TGF-β receptor uses the CME pathway to promote signaling, whereas the caveolae-mediated pathway is used for degradation (11).

TLRs are the most studied germline-encoded pattern recognition receptors, which recognize conserved structures of microbes called pathogen-associated molecular patterns to induce innate and adaptive immunity (12). TLRs are expressed in many immune cells, including macrophages, as well as nonimmune cells such as endothelia (13). Upon activation, TLR4 interacts with MyD88/Toll/IL-1R domain–containing adaptor protein (TIRAP) or Toll/IL-1R domain–containing adaptor inducing IFN-β (TRIF)/TRIF-related adaptor molecule to initiate NF-κB signaling and IFN regulatory factor (IRF) signaling cascade and in turn results in the production of proinflammatory cytokines and type I IFN for induction of inflammatory responses and antiviral responses, respectively (14–16).

TLR4 localizes in the plasma membrane and is usually associated with CD14 and MD2, which are required for maximal LPS activation (16). The proper localization of TLR4 is essential for its signaling, as improper intracellular localization impairs its responsiveness to LPS and is responsible for certain immune disorders and autoimmune diseases (17). Usually, TLR4 endocytosis is through the CME pathway (18, 19), which plays essential roles in activation of specific signal transduction pathways such as induction of IFN-β (19). Tyrosine kinase Syk and phospholipase Cγ2 have been identified as important regulators of TLR4 endocytosis (20). Studies have shown that stimulation with LPS shifts TLR4 to lipid raft–enriched domains, which are required for TLR4 signaling (21–23). This suggests a potential connection between TLR4 function and lipid rafts. However, the nature of this relationship and the mechanisms through which it may work remain to be explored.

Some studies have demonstrated that adenylyl cyclase (AC) activators, such as PGE_2 and forskolin, inhibit NF-κB signaling and IFN-β at both the mRNA and protein level (24, 25), suggesting complicated cross talk between ACs and TLR4 signaling. Whether ACs regulate TLR4 signaling remains to be demonstrated.

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Abbreviations used in this article: AC, adenylyl cyclase; BMDM, bone marrow–derived macrophage; CME, clathrin-mediated endocytosis; EIA, enzyme immunoassay; ELAM-1, endothelial leukocyte adhesion molecule-1; iNOS, inducible NO synthase; IRF, IFN regulatory factor; MβCD, methyl-β-cyclodextrin; MFI, mean fluorescence intensity; siRNA, small interfering RNA; TIRAP, Toll/IL-1R domain–containing adaptor protein; TRIF, Toll/IL-1R domain–containing adaptor inducing IFN-β.

There are nine transmembrane AC isoforms and each with different amino acid sequences, tissue and chromosomal distribution, and regulation (26, 27). The heterogeneity of AC isoforms has been appreciated for some time, but their specific effects on cell physiology are poorly defined due to expression of multiple isoforms in a single type of cell, activation by the same G protein, and a lack of effective isoform-specific drugs. Our previous studies have reported that through the distinct localization of AC isoforms in either lipid raft or non–lipid raft domains, isoforms can be involved in specific signaling pathways and cellular responses (26).

The present study examined the effects of AC isoforms on TLR4 signaling. We found that activation of AC6 shifts the endocytosis of TLR4, normally progressing through the CME pathway, to a lipid raft–mediated pathway. This shift in internalization pathway led to accelerated degradation of TLR4. This novel mechanism highlights how cAMP signaling by a specific AC isoform can negatively regulate TLR4 signaling and function.

Materials and Methods

Reagents and Abs

LPS from *Salmonella* Minnesota R595 was purchased from Sigma-Aldrich (St. Louis, MO). Pam_3CSK_4 was obtained from Invivogen (San Diego, CA). TLR4 Ab (Sa15–21) was purchased from BioLegend (San Diego, CA). All of the other Abs for FACS analysis were from BioLegend. Ab for caveolin-1 was obtained from BD Biosciences (San Jose, CA). Abs for inducible NO synthase (iNOS), β-actin, and AC isoforms were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-mouse and anti-rabbit secondary Abs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Beraprost and butaprost were obtained from Cayman Chemical (Ann Arbor, MI). All other chemicals and reagents were obtained from Sigma-Aldrich.

Cell culture and transfection

RAW 264.7 cells and HEK 293 cells were purchased from American Type Culture Collection (Bethesda, MD) and cultured as described (28). Briefly, RAW 264.7 cells and HEK 293 cells were cultured in DMEM medium supplemented with 10% FBS. HEK 293 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). RAW 264.7 cells were transfected with TransIT-Jurket (Mirus Bio, Madison, WI) according to the manufacturer's instructions.

Bone marrow–derived macrophages (BMDMs) were obtained as described (29). Briefly, cells were isolated by flushing the bone marrow from femurs and tibias and then maintained in DMEM medium supplemented with 20% FBS and 30% L929 supernatant containing CSF. Six days later, adherent macrophages were dissociated and resuspended in DMEM supplemented with 10% FBS. Female C57BL/6 mice (6–8 wk) were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences and kept under specific pathogen-free conditions in the animal center of Shanghai Jiao Tong University School of Medicine (Shanghai, China). All mouse experiments were approved by the Animal Welfare and Ethics Committee of the Shanghai Jiao Tong University School of Medicine. All efforts were made to minimize suffering.

Overexpression of AC4 or AC6 in BMDMs was performed with lentivirus based on pLVX-IRES-zsGreen containing AC4 or AC6 sequence amplified from mouse cDNA. Small interfering RNAs (siRNAs) targeting AC4, AC6, and scrambled siRNAs were purchased from Applied Biosystems. The siRNA for AC4 knockdown was s98218 and for AC6 was s61991. BMDMs were plated in 24-well plates (2×10^5 cells/well) and transfected the next day with 30 pmol siRNA and 2 μl Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h, the siRNA-transfected cells were analyzed or for other experiments.

Measurement of AC activity

AC activities were measured in membranes of RAW 264.7 and BMDM as previously described (26). Briefly, cells were homogenized, centrifuged at low speed, and then the supernatant was transferred to a centrifuge tube and centrifuged at $5000 \times g$ for 10 min. The pellet was suspended and added to tubes containing drug and AC assay buffer. Reactions were stopped by boiling, and cAMP content was measured by enzyme immunoassay (EIA; GE Healthcare). AC activity was normalized to the amount of protein per sample as determined using a dye-binding protein assay (Bio-Rad, Hercules, CA).

Luciferase reporter assay

For luciferase reporter assay, we used lentivirus based on pLenti CMV V5-LUC Blast containing IFN-β or endothelial leukocyte adhesion molecule-1 (ELAM-1) and AC6 or AC4. Recombinant lentivirus was used to infect cells. Twenty-four to 48 h later, the cells were treated with LPS (100 ng/ml) alone or LPS plus forskolin (10 μM) for 6 h, then the cells were lysed, and luciferase activity was determined using reagents from Promega (Madison, WI). Relative luciferase activities were calculated as fold induction compared with an unstimulated vector control. The data are presented as mean ± SEM of at least three independent experiments.

Immunoblotting

Immunoblotting was performed as previously described (30). Cells with or without treatment were collected and lysed in lysis buffer containing 1% Nonidet P-40. Following brief vortexing and rotation, cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. These membranes were blocked with 5% fat-free milk in PBS-Tween 20 and incubated with primary Ab and then with proper HRP-conjugated secondary Ab. After subsequent washes, the immunoreactive bands were detected with ECL plus immunoblotting detection reagents (Amersham Pharmacia Biotech).

RT-PCR

RT-PCR for AC isoforms was performed using the primer pairs (Supplemental Table I). Total RNA was extracted from BMDM cells using TRIzol reagent (Invitrogen), and the RNA was reverse transcribed using Superscript II (Invitrogen) and random hexamer primer. The cDNA was used as template for PCR with AC isoform-specific primer pairs, and PCR products were analyzed by agarose gel electrophoresis and visualized under UV light with ethidium bromide.

Cellular fractionation by sucrose density gradient

Up to 80% confluent cells were rinsed with PBS and lysed in buffer containing 1% Lubrol WX at 4°C. The samples were mixed with an equal volume of 80% sucrose in the same buffer without Lubrol WX and overlaid with a discontinuous sucrose gradient containing 5 ml 30% sucrose and 1 ml 5% sucrose in buffer lacking Lubrol WX. The samples were spun at 35,000 rpm for 20 h at 4°C in a SW41Ti rotor (Beckman Coulter, Fullerton, CA). After the completion of the centrifugation, fractions were collected by sequentially removing 1-ml aliquots from the top.

Nondetergent isolation of lipid raft and non–lipid raft membranes

Cells were fractionated using a detergent-free method. Ten-centimeter dishes at 70–80% confluence were washed twice in ice-cold PBS and scraped into a total of 1.5 ml 500 mM sodium carbonate (pH 11). Cells were homogenized, and 1 ml homogenate was brought to 45% sucrose and loaded in an ultracentrifuge tube. A discontinuous sucrose gradient was layered on top of the sample by placing 2 ml 35% sucrose and then 1 ml 5% sucrose. The gradient was centrifuged at 46,000 rpm on a SW55Ti rotor (Beckman Coulter, Fullerton, CA) for 16–18 h at 4°C. The faint light-scattering band was collected from the 5–35% sucrose interface (lipid raft fractions), and the bottom of the gradient (45% sucrose) was collected as non–lipid raft material. Raft and non–lipid raft fractions, along with whole-cell lysate, were then analyzed by SDS-PAGE and immunoblotting. For AC isoform detection, samples were deglycosylated and concentrated before SDS-PAGE.

Immunoprecipitation

For immunoprecipitation, cell lysates were incubated with the indicated Ab plus protein G-Sepharose in the cold room overnight. After extensive washing with lysis buffer (150 mM NaCl, 50 mM Tris, 0.5 mM EDTA, and 1% Nonidet P-40), the immune complexes were separated by centrifugation and analyzed by immunoblotting as described.

Endocytosis assay

For flow cytometry–based endocytosis assays, we used Ab-probed endocytosis, as previously described (30). Cells were detached, incubated with FITC-conjugated Abs at 4°C for 1 h, and then switched to 37°C for different periods of time for internalization. After acidic washes (0.1 M glycine and 0.1 M NaCl [pH 2.5]), the cells were fixed with 3% paraformaldehyde and subsequently analyzed using an FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). The percentage of internalization was calculated using the formula: (mean fluorescence intensity [MFI] of the internalization at a given time point − MFI of the internalization at time zero)/MFI of the total surface molecules × 100%.

We used cell-surface protein biotinylation to monitor endocytosis, as previously described (30). Cells at ~80% confluence were treated with the indicated treatments, and then 2 ml sulfo-NHS-LC-LC-biotin (1 mg), a membrane-impermeable biotinylation reagent, was added for 1 h at 4°C. Cells were then switched to 37°C for different periods of time for internalization. Cellular extracts were prepared with 200 μl lysis buffer and then incubated with immobilized streptavidin agarose, which was subjected to SDS-PAGE and immunoblot analysis with TLR4 Ab. For some experiments, after internalization, cell lysate was directly subjected to SDS-PAGE and immunoblot analysis with TLR4 Ab. We then quantitatively analyzed the band densitometry using ImageJ software (National Institutes of Health).

Statistical analysis

The two-tailed Student *t* test was used for all statistical analyses in this study. A p value < 0.05 was considered statistically significant.

Results

AC activation attenuates TLR4 signaling stimulated with LPS

AC has been implicated as having a regulatory role in various immune events. Upon activation, AC catalyzes the conversion of ATP to cAMP, which plays key roles in immune cell proliferation and differentiation. Numerous activators of AC increased the intracellular concentration of cAMP, with forskolin treatment inducing the largest response (Fig. 1A). We thus used forskolin as a direct AC agonist in the remainder of our studies. Macrophages express many kinds of pattern recognition receptors and play indispensable roles in innate immunity. As the most well-studied ligand for TLR4, LPS stimulation led to TLR4 activation in macrophages through both MyD88-dependent and -independent pathways. We monitored NF-κB or IRF signal transduction by luciferase assay in the macrophage cell line RAW 264.7 and BMDMs. We found that treatment with forskolin significantly inhibited TLR4 signaling through both the NF-κB and IRF pathways in RAW 264.7 (Fig. 1B) and BMDMs (Fig. 1C). Although TLR2 ligand Pam3CSK4 activates NF-κB and IRF cascades, treatment with forskolin had no considerable effect on NF-κB or IRF signaling ($p > 0.05$) (Fig. 1B, 1C). Consequently, production of cytokines, such as TNF-α and IFN-β, by BMDMs were reduced when LPS was the stimulus, whereas there were no significant effects in cell stimulated with Pam_3CSK_4 (Fig. 1D). As an important downstream product of LPS signaling, iNOS expression, was also increased upon LPS stimulation. AC activation by forskolin also suppressed upregulation of iNOS (Fig. 1E). Together, our results demonstrate that AC activation can dampen the signaling of TLR4 in myeloid cells.

AC6 activation is involved in reducing TLR4 signaling in BMDMs

TLR4 is expressed on the cell surface largely to detect Gram-negative bacteria and numerous other intracellular and extracellular ligands (16, 31). After stimulation with LPS, TLR4 tended to redistribute to detergent-resistant domains (e.g., lipid raft domain)

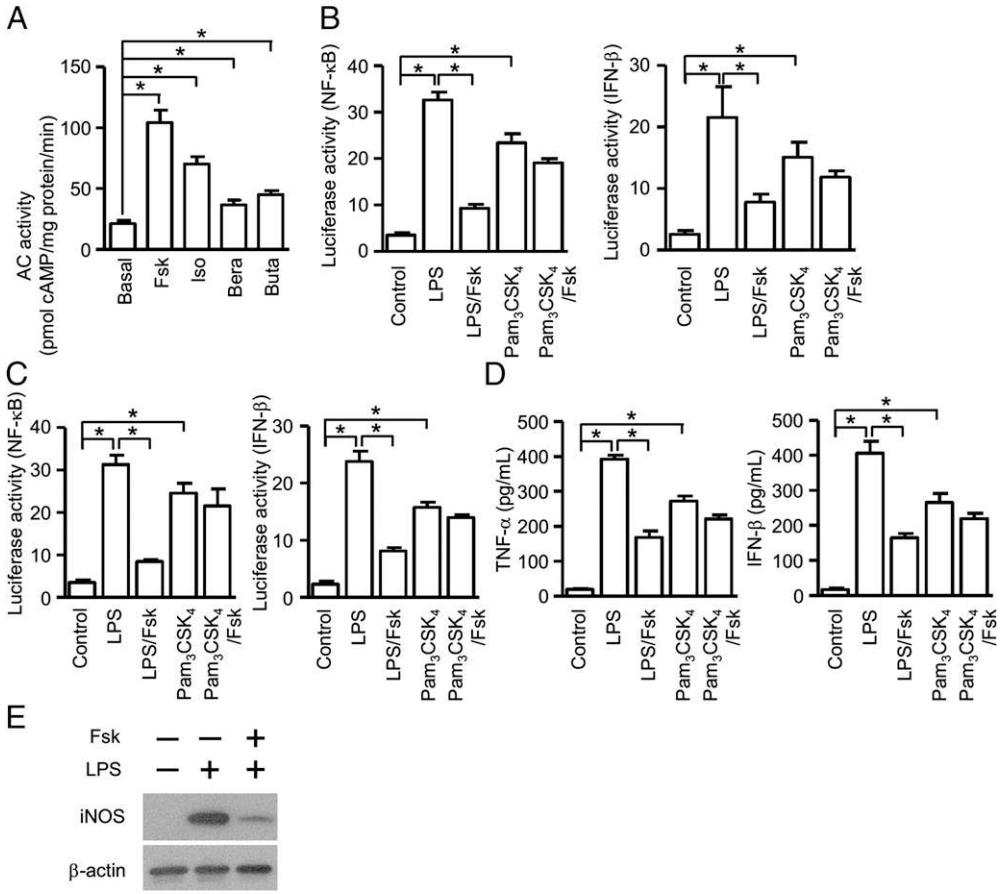

FIGURE 1. AC agonist attenuates TLR4 signaling. (**A**) Membranes were prepared from RAW 264.7 and then incubated with 10 μM forskolin (Fsk), 1 μM isoproterenol (Iso), 1 μM beraprost (Bera), or 1 μM butaprost (Buta) in buffer for 15 min. AC activities were monitored by determining cAMP content by EIA. Data are mean ± SEM of four independent experiments. RAW 264.7 (**B**) or BMDM (**C**) cells were transfected with an ELAM-1 promoter-controlled (*left panel*) or an IFN-β promoter–controlled luciferase reporter gene (*right panel*) and then treated with Fsk (10 μM), LPS (100 ng/ml), or LPS plus Fsk (10 μM) for 6 h. Cells were lysed, and relative luciferase activities were determined. (**D**) Supernatants from BMDM were used to detect the levels of cytokines TNF-α and IFN-β by ELISA. Data are mean ± SEM of three independent experiments (B–D). (**E**) Cell lysates were analyzed by immunoblotting with anti-iNOS Ab for the activation of TLR4 signaling. The images shown are representative of three independent experiments. *$p < 0.05$.

(Fig. 2A), a step that is essential for downstream signaling (19, 20). To further explore the relationship between TLR4 with AC, we first assessed the expression of AC isoforms in BMDMs using RT-PCR. As the AC9 isoform is poorly activated by forskolin, we only examined expression of the other eight isoforms. AC4 and AC6 mRNA were highly expressed in murine BMDMs, whereas lower levels of AC2 were detected (Fig. 2B). Immunoblotting readily detected expression of AC4 and AC6 in BMDMs (Fig. 2C). Using sucrose gradient fractionation, we detected abundant AC4 and AC6 immunoreactivity and very faint immunoreactivity for AC2, but only AC6 was detected in lipid raft domains (Fig. 2D). To investigate the effect of AC6 on TLR4 signaling, we overexpressed AC4 or AC6 in BMDMs. As shown in Fig. 2E, expression of AC4 or AC6 increased with plasmid concentration. Overexpression of AC6 had no effects on exogenous TLR4 expression (Fig. 2E) and TLR4 signaling (Fig. 2F). However, if activated with forskolin, overexpression of AC6 reduced TLR4 signaling (Fig. 2F) and the release of multiple cytokines (Fig. 2G). To clarify whether this effect of AC6 overexpression is specific to TLR4 signaling, we performed the same experiments on TLR2. The data show that the overexpression of AC6 has no detectible effect on TLR2 signaling and cytokine release (Supplemental Fig. 1A, 1B). Taken together, these data demonstrate that AC6 activation specifically reduces TLR4 signaling in BMDMs.

Fsk treatment shifts TLR4 endocytosis to lipid raft–mediated pathway and promotes its degradation

Endocytosis is a major pathway to negatively regulate the expression of membrane proteins. For TLR4, proper endocytosis is essential for its two distinct signaling pathways involving MyD88 and TRIF. Loss of surface expression is usually used as a readout for membrane protein endocytosis. We used highly sensitive assays to monitor TLR4 internalization by flow cytometry and membrane biotinylation. Because Ab exposure has the potential to alter the kinetics of TLR4 endocytosis, we further used biotin to label the membrane protein and to examine the loss of surface TLR4 by immunoblotting. Stimulation of murine BMDM with LPS induced TLR4 endocytosis through the CME pathway, as defined by its potassium sensitivity and dynamin dependence (Fig. 3A, 3B). Dynasore is a highly specific inhibitor of dynamin, a GTPase that regulates CME. Addition of forskolin recovered the internalization of TLR4 by LPS, even when dynamin was inhibited (Fig. 3B). Depletion of

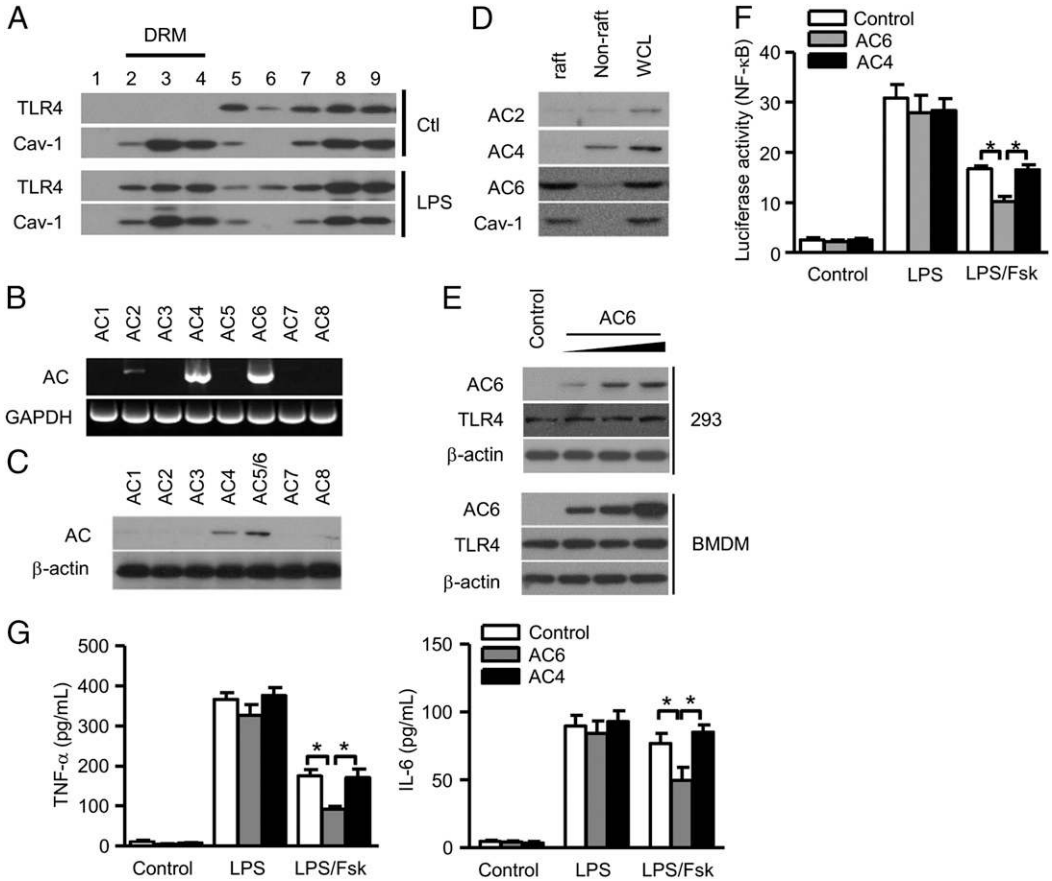

FIGURE 2. AC6 is involved in suppressing TLR4 signaling. (**A**) BMDMs were treated with LPS (100 ng/ml) for 6 h, then lysed, and fractionated as described in *Materials and Methods*. The light-to-heavy fractions were designated as fractions 1–9. Fractions were analyzed by immunoblotting using TLR4 Ab. (**B**) Total RNA was extracted from BMDM, reverse-transcribed, and then PCR performed using AC isoform–specific primer pairs. The image is representative of three experiments. (**C**) Cell lysates from BMDM were analyzed by immunoblotting with AC isoform–specific Abs for the expression of AC isoforms. (**D**) AC isoforms expressed in lipid raft, non–lipid raft, or whole-cell lysate (WCL) fractions from BMDM were analyzed by immunoblotting with isoform-specific Abs. Images shown are representative of three experiments. (**E**) BMDMs were transfected with Myc-AC4 (0.1, 0.3, and 1.0 μg, *top panel*) or Myc-AC6 (0.1, 0.3, and 1.0 μg, *bottom panel*) plasmid, then lysed to perform SDS-PAGE, and immunoblotted with anti-Myc and anti-TLR4 Abs. Images shown are representative of three independent experiments (A–E). (**F**) BMDMs were infected by lentivirus containing luciferase reporter gene and ELAM-1 or IFN-β and AC6 or AC4 with a multiplicity of infection of 10. Twenty-four to 48 h later, the cells were treated with LPS (100 ng/ml) alone or LPS plus forskolin (Fsk; 10 μM) for 6 h. Supernatants were collected, and the cells were lysed before measurement of luciferase activity. (**G**) TNF-α and IL-6 levels were detected in cell supernatants using ELISA. The results represent the mean ± SEM of three independent experiments (F, G). *$p < 0.05$. DRM, Detergent-resistant membrane.

plasma membrane cholesterol with methyl-β-cyclodextrin (MβCD) blocked this forskolin-induced endocytosis, suggesting lipid rafts are necessary for this response (Fig. 3B). Because MβCD treatment could change the expression of membrane proteins, we measured the surface staining of TLR4. Our results show that MβCD treatment has no significant effect on the amount of surface TLR4, either in the presence of LPS or LPS/Fsk (Fig. 3C). Cell-surface protein biotinylation assays also indicated that AC6 activation initiates lipid raft–mediated endocytosis, which accelerates TLR4 degradation (Fig. 3D). TLR4 degradation was reversed if MβCD was used to block lipid raft–mediated endocytosis of surface TLR4. These data are consistent with the idea that TLR4 can be endocytosed through different pathways and that Fsk treatment can shift the route of internalization of TLR4 and alter its downstream signaling.

AC6 activation enhances its interaction with TLR4 in a lipid raft–enriched domain of plasma membrane

In the resting state, AC6 resides primarily in lipid raft–enriched domains. Even when activated by agonist treatment, there was no visible redistribution of AC6 (Fig. 4A). Because LPS treatment shifts TLR4 distribution to the lipid raft–enriched domains, we performed immunoprecipitation to determine if this translocation facilitates a direct interaction between TLR4 and AC6. Without treatment with LPS, there was no detectible interaction between TLR4 and AC6, or TLR4 and AC4, as assessed by immunoprecipitation (Fig. 4B). Following LPS treatment, which induced TLR4 translocation to lipid raft–enriched domains, there was still no interaction detected between AC6 and TLR4. However, treatment with LPS and forskolin led to an interaction between TLR4 and AC6, as detected by coimmunoprecipitation (Fig. 4B). Lipid raft

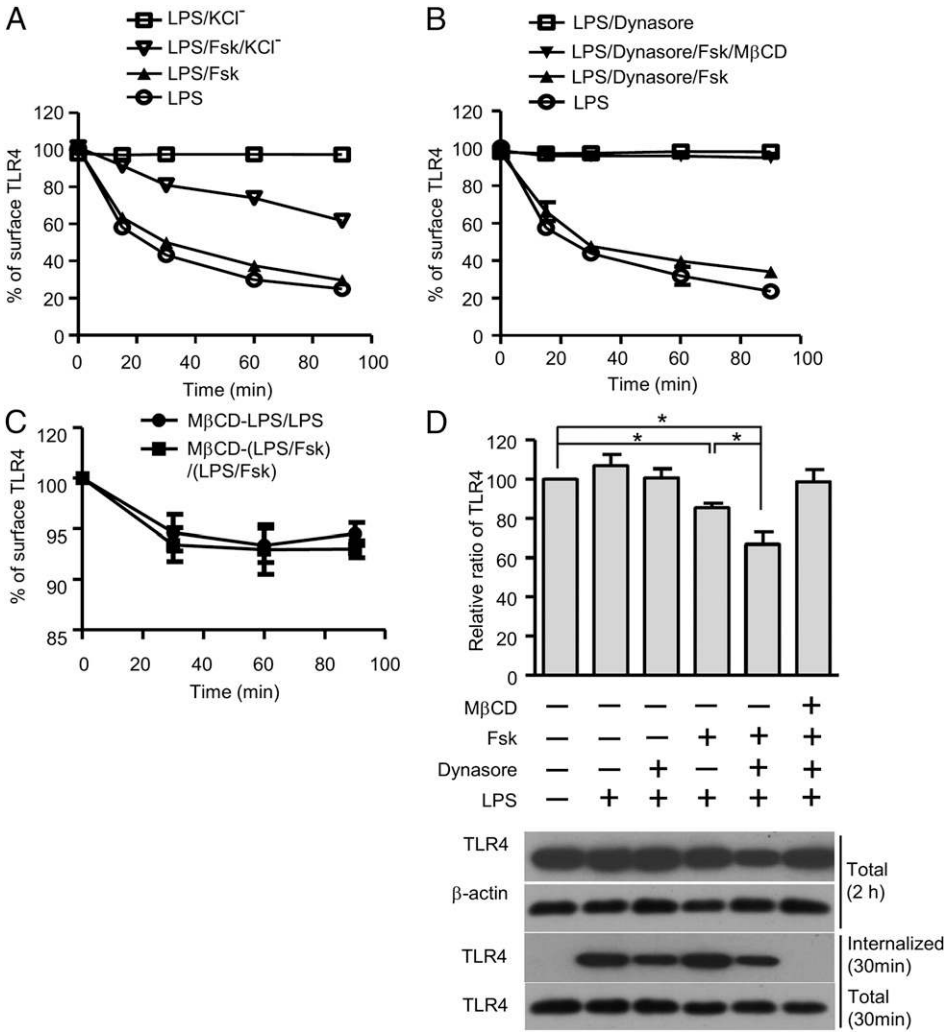

FIGURE 3. AC6 activation shifts endocytosis of TLR4 to the lipid raft–mediated pathway. (**A**) BMDM were untreated or treated with LPS (100 ng/ml) alone, LPS plus forskolin (Fsk; 10 μM), and/or with K^+-free isotonic buffer for the times indicated. After acidic washes, the cells were fixed and analyzed with flow cytometry. The internalization rates were calculated with the formula as described in *Materials and Methods*. (**B**) BMDM were pretreated with or without 5 μM MβCD for 60 min and then untreated or treated with LPS (100 ng/ml) alone, LPS plus Fsk (10 μM), and/or Dynasore (50 μM) for the times indicated. After acid washes, the cells were fixed and analyzed using flow cytometry. The internalization rates were calculated with the formula mentioned above. (**C**) BMDM were pretreated with or without 5 μM MβCD for 60 min and then untreated or treated with LPS (100 ng/ml) alone or LPS plus Fsk (10 μM) for the times indicated. After staining with FITC-TLR4, the cells were fixed and analyzed with flow cytometry. (**D**) After proper treatment as indicated, BMDMs were treated with 1 mg ice-cold sulfo-NHS-LC-LC-biotin for 1 h at 4°C and then switched to 37°C for 30 min to induce internalization. Cellular extracts were prepared with 200 μl of lysis buffer and then incubated with immobilized streptavidin agarose, which was subjected to SDS-PAGE and immunoblot analysis with TLR4 Ab. Some cells were left to internalize for 2 h, and cell lysate was directly subjected to SDS-PAGE and immunoblot analysis with TLR4 Ab. The *top panel* represents densitometry quantitation of total TLR over β-actin using ImageJ. Shown is a representative image of three experiments (D), and the quantitative data are presented as the mean ± SEM of three experiments (A–D). $*p < 0.05$.

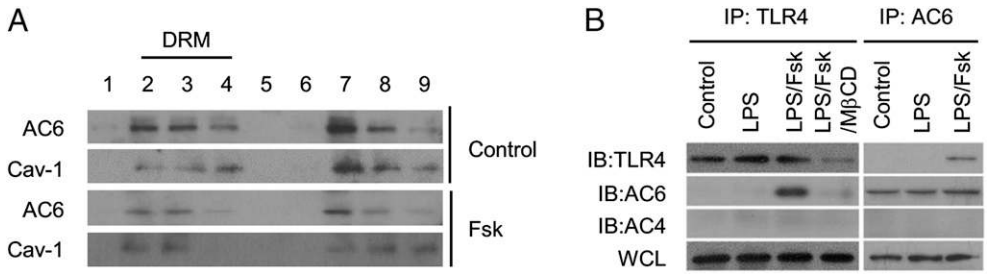

FIGURE 4. AC6 activation is required for interaction with TLR4. (**A**) BMDM cells were treated with forskolin (Fsk; 100 ng/ml) for 6 h and then lysed and fractionated by sucrose gradient centrifugation. The light-to-heavy fractions are designated as fractions 1–9. Fractions were analyzed in immunoblotting using AC6 Ab. Images shown are representative of three experiments. (**B**) BMDMs were treated without or with LPS (100 ng/ml) alone, in combination with Fsk (10 μM), or with Fsk and MβCD (5 μM) for 6 h. BMDM lysates were immunoprecipitated (IP) with Ab against TLR4 or AC6, followed by immunoblotting (IB) for the presence of TLR4, AC6, and AC4 in the immune complexes. Images shown are representative of three experiments. DRM, Detergent-resistant membrane.

disruption by MβCD treatment abrogated this interaction, suggesting that lipid raft domains are necessary for AC6 interaction with TLR4 (Fig. 4B). Thus, activation of AC6 causes it to interact with TLR4 in lipid raft domains of the plasma membrane.

Suppressive effects of AC6 agonist on inflammatory cytokine production

To further confirm a role for AC6 in the regulation of TLR4 signaling, we knocked down the native expression of ACs in BMDM using siRNA. As shown in Fig. 5A, the expression of AC6 or AC4 was significantly reduced by the corresponding siRNA but not by scrambled siRNA. There was no nonspecific nor compensatory changes in either isoform due to siRNA treatment. In BMDM with AC6 or AC4 knockdown, total cellular cAMP production in basal or forskolin-stimulated conditions was similar to control cells (Fig. 5B). Presumably other AC isoforms expressed in BMDMs provided redundant capacity for Gs-stimulated cAMP synthesis. AC6 knockdown did not affect LPS-stimulation of NF-κB activity, but completely reversed the inhibitory effect of forskolin on TLR4 activation and downstream release of multiple inflammatory cytokines, leading to no significantly statistical difference compared with LPS treatment alone (Fig. 5C, 5D). We also performed a series of experiments on TLR2 activation, and our results show that AC6 knockdown has no considerable effect on TLR2 signaling and cytokine release (Supplemental Fig. 1C, 1D). Thus, AC6 specifically effects TLR4 signaling over other receptor pathways. In addition, AC6 is uniquely qualified for these roles, as AC4 knockdown had no significant effect on TLR4 signaling. These data are consistent with the hypothesis that AC6 has specific roles in regulation of TLR4 endocytosis over other AC isoforms.

Discussion

After recognition of microbial products, TLRs initiate innate immune responses and adaptive immunity. However, the binding of self-molecules by TLRs and impaired dampening of TLR signaling also lead to some serious diseases. TLR4 is one of the prime

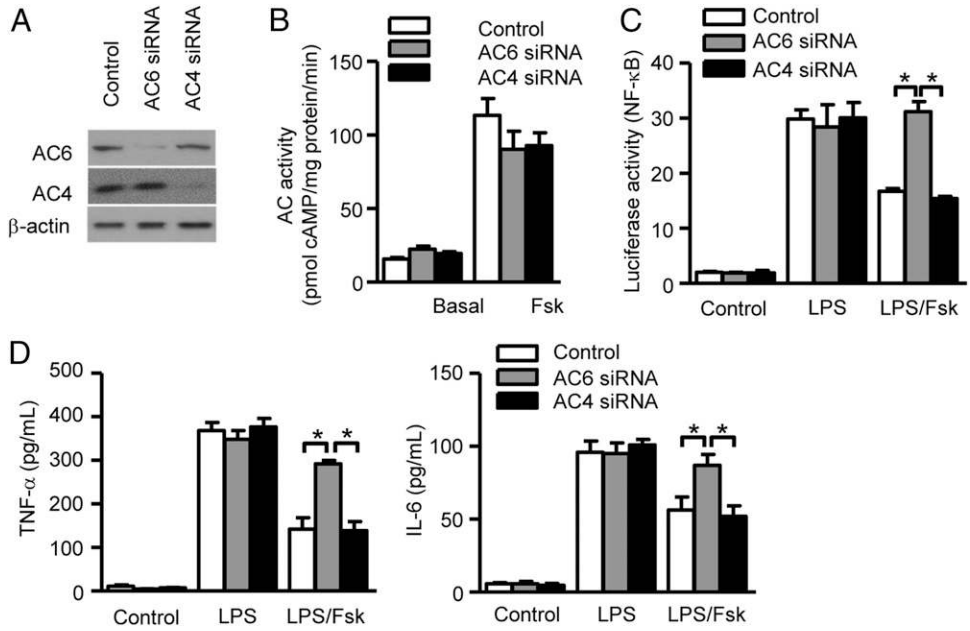

FIGURE 5. Knockdown of AC6 abolishes suppressive effects on TLR4 signaling. (**A**) Suppression of AC6 or AC4 expression by siRNA in BMDMs. Specific siRNA sequences were transfected into BMDMs, and the efficiency of siRNA in inhibition of AC6 or AC4 expression was determined using immunoblotting. The images shown are representative of three independent experiments. (**B**) Membranes were prepared from BMDM with AC6 or AC4 knockdown, untreated or treated with LPS and/or forskolin (Fsk), and then AC activities were monitored by determining cAMP content using EIA. (**C**) BMDMs with AC6 or AC4 knockdown, untreated or treated with LPS and/or Fsk, were transfected with an ELAM-1 promoter–controlled luciferase-reporter gene, then were lysed, and relative luciferase activities were determined. (**D**) Culture medium from BMDMs was used to detect the levels of cytokines TNF-α and IL-6 using ELISA. Data are mean ± SEM of three independent experiments (B–D). *$p < 0.05$.

examples of the importance of the TLR family, as its signaling is critical for host defense and homeostasis. Because appropriate responses and self-tolerance require correct TLR compartmentalization, it is understandable that understanding the endocytosis of TLR4 has attracted much interest of researchers (17, 20, 32). It has been clearly shown that TLR4 is internalized through the CME pathway, and endocytosis of TLR4 coordinates the activation of the TIRAP–MyD88 and TRIF-related adaptor molecule–TRIF signaling pathways (17). Besides CD14, tyrosine kinase Syk and phospholipase Cγ2 also have been identified as important regulators of TLR4 endocytosis (20). The p110δ isoform of PI3K acts as a balance between pro- and anti-inflammatory TLR4 signaling in dendritic cells through compartmentalization mediated by TIRAP-anchoring plasma-membrane lipid phosphatidylinositol-(4,5)-bisphosphate (32).

Our present data show regulation of TLR4 signaling by another pathway, AC and cAMP. Overexpression of AC6 in BMDMs has little effect on LPS-induced TLR4 signaling, indicating little basal activity of this AC isoform. However, activation of AC6 with forskolin dampens TLR4-mediated signaling and response. TLR4 signaling involves several downstream pathways, such as the NF-κB, IRF, and MAPK cascades (14, 15, 33). It has been shown that both NF-κB and MAPK pathways are involved in production of TNF-α and IL-6 (34, 35), although the size of effect on these different cytokines is arguable. Actually, our separate studies demonstrated that in smooth muscle cells, the inhibition of IL-6 by cAMP can only occur from specific AC isoforms that participate in distinct cAMP signaling compartments (A.S. Bogard, A.V. Birg, and R.S. Ostrom, submitted for publication). So we believe that Fsk can only inhibit IL-6 when the proper cAMP compartment is activated, whereas TNF-α is effected by any cAMP signal in the cell. This could be the reason why we observed less reduction of IL-6 secretion by LPS/forskolin but a sizeable effect on both TNF-α and IFN-β secretion.

As has been reported, TLR4 endocytosis is normally through the CME pathway, which is essential for its signaling. Our data show that upon potassium depletion and Dynasore treatment, which block CME endocytosis, internalization of membrane TLR4 still occurs when LPS-stimulated BMDMs are treated with forskolin. Depletion of plasma membrane cholesterol with MβCD abrogates forskolin-induced endocytosis, confirming the role of a lipid raft–mediated pathway. We reason that AC activation initiates lipid raft–dependent endocytosis, which most likely inhibits CME, and accelerates degradation of TLR4. Thus, different pathways may play different roles: CME may be essential for TLR4 signaling, whereas the lipid raft–mediated pathway is central for TLR4 turnover, providing an efficient means for protein degradation. Our studies reveal a new mechanism of negative regulation of TLR4 turnover and signaling, which enhances our knowledge of this regulatory network for innate immunity signaling.

ACs associate with G protein–coupled receptors in signaling microdomains in the plasma membrane to play specific roles in diverse biological events (36, 37) including modulation of innate and adaptive immunity (38). AC7 controls the extent of immune responses toward bacterial infection and is required for optimal functions of B and T cells (39). Some pathogens use adenylate cyclase toxin to manipulate the host's immune system (40–42). The parasite *Trypanosoma brucei* has evolved a large family of transmembrane receptor-like adenylate cyclases, for which activation inhibits the synthesis of the trypanosome-controlling cytokine TNF-α through activation of protein kinase A, favoring its early colonization (43).

This study uncovers a role for AC6 in regulating TLR4 endocytosis and maintaining immune homeostasis. These findings have implications for treatment of various inflammatory and autoimmune diseases. TLR4 activation leads to signaling by several downstream pathways, such as NF-κB and IRFs, which are involved in diverse processes including pathogen clearance. Without proper regulation, these mechanisms inflict serious damage, and even death, on the host. Improper activation of TLR4 contributes to inflammatory diseases such as acute lung injury (44) and autoimmune diseases like rheumatoid arthritis (45). It is no wonder that hosts balance TLR4 effects to ensure both pathogen clearance and host health. Negative regulation of TLR4 is a field of intense research (46), and our findings uncover a role for a specific AC isoform, AC6, in modulating innate immunity.

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Disclosures

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