MicroRNA-33 Regulates the Innate Immune Response via ATP Binding Cassette Transporter-mediated Remodeling of Membrane Microdomains*

Received for publication, February 19, 2016, and in revised form, July 18, 2016. Published, JBC Papers in Press, July 28, 2016, DOI 10.1074/jbc.M116.723056

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MicroRNAs (miRNAs) are short non-coding RNAs that regulate gene expression by promoting degradation and/or repressing translation of specific target mRNAs. Several miRNAs have been identified that regulate the amplitude of the innate immune response by directly targeting Toll-like receptor (TLR) pathway members and/or cytokines. miR-33a and miR-33b (the latter present in primates but absent in rodents and lower species) are located in introns of the sterol regulatory element-binding protein (SREBP)-encoding genes and control cholesterol/lipid homeostasis in concert with their host gene products. These miRNAs regulate macrophage cholesterol by targeting the lipid efflux transporters ATP binding cassette (ABC)A1 and ABCG1. We and others have previously reported that Abca1⁻/⁻ and Abcg1⁻/⁻ macrophages have increased TLR proinflammatory responses due to augmented lipid raft cholesterol. Given this, we hypothesized that miR-33 would augment TLR signaling in macrophages via a raft cholesterol-dependent mechanism. Herein, we report that multiple TLR ligands down-regulate miR-33 in murine macrophages. In the case of lipopolysaccharide, this is a delayed, Toll/interleukin-1 receptor (TIR) domain-containing adapter-inducing interferon-β-dependent response that also down-regulates Srebf-2, the host gene for miR-33. miR-33 augments macrophage lipid rafts and enhances proinflammatory cytokine induction and NF-κB activation by LPS. This occurs through an ABCA1- and ABCG1-dependent mechanism and is reversible by interventions upon raft cholesterol and by ABC transporter-inducing liver X receptor agonists. Taken together, these findings extend the purview of miR-33, identifying it as an indirect regulator of innate immunity that mediates bidirectional cross-talk between lipid homeostasis and inflammation.

MicroRNAs (miRNAs)³ are small (~22-nucleotide) non-coding RNAs that regulate gene expression by binding to partially complementary sites in the 3’-untranslated regions (UTRs) of specific mRNAs, thereby promoting degradation and/or repressing translation of target mRNAs. The human genome contains >2500 unique mature miRNAs (1). Because individual miRNAs typically have multiple targets, it is thought that >60% of all human genes may be subject to regulation by miRNAs (2). The promiscuity of miRNAs for target RNAs is expected to represent a fundamental mechanism of cross-talk and coordination among signaling networks in development, health, and disease.

Among the signaling networks that have been shown in recent years to be regulated by miRNAs are the Toll-like receptors (TLRs) of the innate immune system. Multiple TLRs up-regulate miRNAs, including miR-155, miR-146, miR-21, miR-147, and miR-9, whereas activation of TLR4 by lipopolysaccharide (LPS) down-regulates a distinct set of miRNAs (3, 4). In turn, miRNAs “fine tune” the proinflammatory signaling output of TLR cascades by controlling the expression of TLR pathway members (3, 4). Thus, miR-146 suppresses signaling by multiple TLRs via targeting the common signaling hubs IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6 (5), whereas miR-155 has complex effects, repressing the TLR adaptor myeloid differentiation primary response 88 (MyD88) (6, 7) and TAK1-binding protein 2 (8), an upstream activator of the mitogen-activated protein kinases, but also promoting cytokine expression through actions on other targets (3). Although virtually all known examples of TLR regulation by miRNAs operate through direct targeting of TLR pathway components, it is expected that miRNAs may also indirectly impact the innate immune response by regulating other networks that cross-talk with TLRs.

miR-33a and miR-33b (present in primates but absent in rodents and lower species in which miR-33a is simply referred to as “miR-33”) are now known to be master regulators of cho-

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1 This work was supported in part by Intramural Research Program, NIEHS, National Institutes of Health, and the NIH National Institute of Environmental Health Sciences, 111 T. W. Alexander Dr., P. O. Box 12233, MD D2-01, Research Triangle Park, NC 27709. Tel.: 919-541-3701; Fax: 919-541-4133; E-mail: fesslerm@niehs.nih.gov.

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3 The abbreviations used are: miRNA, microRNA; miR, microRNA; ABC, ATP binding cassette; LXR, liver X receptor; MyD88, myeloid differentiation primary response 88; SREBP, sterol response element-binding protein; TLR, Toll-like receptor; TRIF, Toll/interleukin-1 receptor (TIR) domain-containing adapter-inducing interferon-β; 25HC, 25-hydroxycholesterol; PEM, peritoneal elicited macrophage; qRT-PCR, quantitative RT-PCR.
lesterol homeostasis. Both miRNAs target ATP binding cassette transporter A1 (ABCA1) (9–11), a protein that plays a central role in cellular cholesterol efflux and in biogenesis of high density lipoprotein (HDL). These miRNAs are embedded in introns of, and co-transcribed with, genes encoding the sterol regulatory element-binding proteins (SREBPs; SREBF1 (encodes SREBP-1) for miR-33b and SREBF2 (encodes SREBP-2) for miR-33a) (9–11). SREBPs are transcription factors that are induced during cellular lipid deficit and that up-regulate genes involved in cholesterol and fatty acid synthesis and trafficking. By reducing cellular cholesterol efflux via repression of ABCA1 (and ABCG1 in rodents), miR-33 collaborates with SREBPs to elevate cellular cholesterol levels. We and others have reported that ABCA1 and ABCG1 negatively regulate signaling by TLRs by depleting cholesterol-enriched lipid raft membrane microdomains in which TLR complexes are assembled and activated (12, 13). Thus, both Abca1-null and Abcg1-null murine macrophages have augmented lipid rafts and enhanced proinflammatory TLR signaling that may be normalized by raft cholesterol-reducing agents, and macrophages dually deficient in both ABC proteins have a further accentuation of this phenotype (13). Given that miR-33 represses both ABCA1 and ABCG1, we hypothesized that it would enhance TLR signaling in macrophages through a raft cholesterol-dependent mechanism.

Herein, we report that miR-33 is down-regulated by multiple TLR ligands in murine macrophages. miR-33 augments macrophage lipid rafts and enhances proinflammatory responses to LPS. This occurs through an ABCA1- and ABCG1-dependent mechanism and is reversible by raft cholesterol-reducing agents and ABC transporter-inducing liver X receptor (LXR) agonists. Taken together, these findings newly identify miR-33 as an indirect regulator of innate immunity.

Results

miR-33 Expression Is Down-regulated by Multiple TLR Pathways—Several miRNAs are regulated in response to different TLR ligands (3). miR-33 is up-regulated by low sterol conditions, including lipid deprivation and statin treatment, and down-regulated by lipid loading (9–11), but its sensitivity to TLR ligands has not previously been reported to our knowledge. We thus profiled expression levels of miR-33 and its host gene, Srebf-2, at different time points after LPS in primary murine macrophages. A modest time-dependent up-regulation of Srebf-2 was seen within the first 4 h of LPS stimulation followed by a more dramatic down-regulation at 16–48 h (Fig. 1A). Although coordinate down-regulation of miR-33 was observed at 16–48 h, there was no consistent effect on miR-33 expression at early times post-LPS (<8 h) (Fig. 1B). After transcription, primary miRNAs are processed by Drosha and then
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by Dicer into mature miRNAs. Discordant regulation between Srebf-2 and miR-33 suggested to us that post-transcriptional processing of the latter might be suppressed and/or delayed at early time points after LPS. Consistent with this, we found that, like Srebf-2, LPS robustly up-regulated primary miR-33 at times <4 h postexposure (Fig. 1C). Consistent with past reports (14, 15), we also found that the early up-regulation of Srebf-2 is NF-κB-dependent as it was attenuated by pretreatment with an NF-κB inhibitor (Fig. 1D).

Similar down-regulation of Srebf-2 and miR33 was seen at late time points of LPS exposure in RAW 264.7 macrophages (Fig. 1E). A concentration dependence of LPS down-regulation of miR-33 at 24 h was also observed (Fig. 1F). Similar to a past report (16), we found that LPS robustly down-regulates Abcg1 in primary macrophages by 4 h and in a sustained fashion out to 24 h (Fig. 1G). By contrast, LPS modestly up-regulated Abca1, including at early time points preceding miR-33 down-regulation. Our finding that LPS down-regulates miR-33 and only later than 8 h postexposure suggests that miR-33 does not mediate the dynamic regulation of Abcg1 by LPS observed in macrophages.

SREBP-2 is a key lipogenic transcription factor that induces the enzymes of the mevalonic acid pathway (i.e. cholesterol) biosynthesis pathway (17). To test the functional significance of late phase LPS-induced down-regulation of Srebf-2, we thus profiled the mevalonic acid pathway. LPS dramatically down-regulated all six enzymes of the cholesterol synthesis pathway that we surveyed, enzymes that collectively span the full synthetic cascade from acetoacetyl-CoA to cholesterol (Hmgcs1, Hmgcr, Lss, Mvd, Dhc24, and Dhc7) (Fig. 2, A–F). Moreover, consistent with an associated reduction in de novo cholesterol biosynthesis, we found that macrophage free cholesterol mass was significantly reduced 24 h after LPS exposure (Fig. 2G).

The adaptor protein MyD88 is reported to transduce an early pathway of signaling downstream of TLR4 activation and the adaptor TIR domain-containing adaptor-inducing interferon-β (TRIF), a late pathway (18). Given the delayed nature of miR-33 regulation after LPS, we hypothesized that it was a TRIF-dependent response. Consistent with this, we found that down-regulation of miR-33 and Srebf-2 at 24 h after LPS was preserved in Myd88-null macrophages but abolished/attenuated in Trif-null macrophages (Fig. 3A).

Recently, it was reported that LPS up-regulates the interferon-stimulated gene cholesterol 25-hydroxylase (Ch25h) and its lipid product, 25-hydroxycholesterol (25HC), through inducing autocrine IFNβ signaling downstream of TRIF (19, 20). It is well known that 25HC inhibits SREBP-2 activation and thereby also reduces Srebf-2 expression through Srebf-2 autoregulation (21). Given this, we speculated that an IFNβ-Ch25h-25HC axis downstream of TRIF might mediate Srebf-2 down-regulation at late time points after LPS. We confirmed that LPS induces Ch25h in a TRIF- and type I interferon receptor (Ifnar1)-dependent manner in macrophages (Figs. 3B). IFNβ treatment was sufficient to induce robust up-regulation of Ch25h in wild type macrophages and down-regulated both Srebf-2 and miR-33 (Fig. 3C). Similarly, 25HC treatment of wild type macrophages down-regulated both Srebf-2 and miR-33 (Fig. 3D). Lastly, we confirmed that LPS-induced down-regulation of Srebf-2 was attenuated in Ch25h−/− macrophages (Fig. 3E). Taken together, these findings support a contributory role for endogenous Ch25h-dependent 25HC in TRIF-dependent down-regulation of Srebf-2 in macrophages but also indicate that additional mechanisms are likely at play.

We also found that miR-33 was down-regulated 24 h after stimulation with polynosinic:polycytidylic acid (poly(I:C)), a ligand for the TRIF-restricted endosomal TLR, TLR3 (Fig. 4A). miR-33 was also down-regulated at late time points following stimulation with peptidoglycan, a bacterial stimulus of both TLR2 and the MyD88- and TRIF-independent receptor nucleotide oligomerization domain 1 (Fig. 4B) (22, 23). miR-33 was also down-regulated after stimulation with heat-killed Listeria monocytogenes, a stimulus for TLR2, a receptor reported to
induce both MyD88- and TRIF-dependent signaling (Fig. 4C) (18, 24). Taken together, multiple pathogen recognition receptors down-regulate miR-33 at late time points after activation. In the case of TLR3 and TLR4, this occurs through a MyD88-independent mechanism, whereas miR-33 down-regulation by TLR2 ligands may point to the existence of additional regulatory pathways.

miR33 Enhances Proinflammatory Macrophage Responses to Multiple TLR Ligands—Consistent with past reports that miR-33 represses ABCA1 and ABCG1 in murine macrophages (9–11), we confirmed that transfection of a miR-33 mimic oligonucleotide into primary murine macrophages suppressed Abca1 and Abcg1 expression at the mRNA and protein levels, whereas transfection of a miR-33 inhibitory oligonucleotide derepressed (up-regulated) these two targets (Fig. 5, A and B). The latter result suggests a physiologic role for miR-33 in regulation of ABCA1 and ABCG1 expression in the steady state. Given that deficiencies of ABCA1 and ABCG1 are reported to enhance signaling by multiple TLRs (13, 25), we speculated that miR-33 overexpression would do the same. To address this, we transfected macrophages with a miR-33 mimic and then exposed them to a panel of TLR ligands. Consistent with our hypothesis, the miR-33 mimic enhanced LPS induction of both TNFα and IL-6 mRNA and secreted protein (Fig. 5, C and D).
Similarly, the miR-33 mimic enhanced TNFα and IL-6 induction by poly(I:C) and peptidoglycan (Fig. 5E). Consistent with a physiologic role for endogenous miR-33 in promotion of pro-inflammatory cytokine induction by multiple TLRs, transfection of the miR-33 inhibitor suppressed TNFα and IL-6 induction by LPS, poly(I:C), and peptidoglycan (Fig. 5D). E, PEMs transfected as in C and D were exposed (4 h) to poly(I:C) (20 μg/ml) or peptidoglycan (PGN) (10 μg/ml). TNFα and IL-6 protein in the cell medium were determined by ELISA. F and G, PEMs were transfected with inhibitor control or miR-33 inhibitor and then exposed to LPS (100 ng/ml) as shown (F) or to poly(I:C) (20 μg/ml) or peptidoglycan (10 μg/ml) (G) for 4 h. TNFα and IL-6 protein in the cell medium were measured by ELISA. H, PEMs were transfected with control or miR-33 mimic and then stimulated with LPS for different durations as indicated. Cell lysates were analyzed by immunoblotting for the targets shown. Tubulin serves as a loading control. Data are mean ± S.E. (error bars) of a representative experiment of three independent experiments. *, p < 0.05; **, p < 0.01. IKK, IκB kinase.
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FIGURE 6. miR-33 augments lipid raft formation and raft-dependent signaling. A, PEMs were transfected with control (Cont) or miR-33 mimic (40 nM for 24 h) and then left unstained or stained with Alexa Fluor (AF)-tagged cholera toxin B subunit (CTB) to probe raft-associated cell surface gangliosides. Cholera toxin B signal was then quantified by flow cytometry. A representative histogram is shown at left, and mean fluorescence intensity (MFI) of replicate samples is shown at right. B and C, PEMs were transfected with control or miR-33 mimic (40 nM for 24 h) and then treated as shown with vehicle, filipin (6 μg/ml for 1 h), nystatin (50 μg/ml for 1 h), or methyl-β-cyclodextrin (mBCD) (10 mM for 0.5 h). The cells were then exposed to LPS (10 ng/ml for 4 h). TNFα (B) and IL-6 (C) in the cell medium were determined by ELISA. Data are mean ± S.E. (error bars) of a representative experiment of at least three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

(13). We also found that treatment of macrophages with any of several membrane cholesterol-reducing agents that attenuate raft-dependent signaling, including cholesterol-sequestering (filipin and nystatin) and –extracting (methyl-β-cyclodextrin) compounds (12, 13, 26), abolished the enhancement of LPS-induced TNFα and IL-6 observed in miR-33 mimic-treated cells (Fig. 6, B and C). Together, these findings suggest that miR-33 overexpression enhances LPS induction of proinflammatory cytokines via a mechanism involving augmentation of cholesterol-enriched membrane microdomains.

Similar to past reports (13, 25), we found that Abca1-null and Abcg1-null macrophages, which have augmented lipid rafts, produced higher levels of TNFα and IL-6 than wild type macrophages after LPS stimulation with the latter genotype tending to have the higher response of the two (Fig. 7A). Of interest, the effect of miR-33 mimic to enhance LPS induction of both TNFα and IL-6 in wild type macrophages was abolished both in macrophages deficient in Abca1 and in macrophages deficient in Abcg1 (Fig. 7A). This finding suggests that the proinflammatory effect of miR-33 requires expression of both ABC transporters and thus may operate through dual repression of both. Abca1 and Abcg1 are both target genes of the nuclear receptor LXR and are well known to be robustly up-regulated by LXR agonists (27). Consistent with miR-33 enhancing LPS induction of proinflammatory cytokines via repression of ABCA1 and ABCG1, we found that the synthetic LXR agonist TO901317 (28) abolished miR-33 mimic-induced augmentation of TNFα and IL-6 expression (Fig. 7B) under conditions in which it was confirmed to rescue miR33-induced ABC transporter silencing (Fig. 7C). As miR-33 mimic treatment also reduced ABC transporter expression in the setting of TO901317 (but within a supraphysiologic range of expression) (Fig. 7C), we speculate that LXR-driven overexpression of ABCA1 and ABCG1 may “disrupt” rafts from a functional TLR signaling standpoint, making them no longer susceptible to miR-33. Alternatively, it is possible that LXR activation may abolish raft-dependent effects of miR-33 on TLR-induced cytokines by acting downstream of lipid rafts, such as through LXR sumoylation at proinflammatory gene promoters (29).

Discussion

Several miRNAs are regulated by TLRs and in turn modulate TLR pathway signaling output via direct repression of TLR pathway components. Among these, miR-155, miR-146, and miR-21 have been the best studied to date (4). Here, we add miR-33 to the list of miRNAs that are down-regulated by TLRs, in this case, TLR2, TLR3, and TLR4, and show that LPS down-regulates miR-33 through a delayed, TRIF-dependent mechanism. This distinguishes it from miR-155 (rapidly down-regulated and then up-regulated by LPS via either MyD88 or TRIF (30, 31)) and miR-147 (up-regulated by LPS through a mechanism requiring both MyD88 and TRIF (32)). Late LPS-induced regulation of miR-33 is unlikely to impact early signaling events elicited by LPS. Moreover, the miR-33 target ABCG1 is robustly down-regulated by LPS as an early and sustained response, suggesting that this occurs through a miR-33-independent mechanism. Nonetheless, we speculate that down-regulation of miR-33 may still possibly feed back to act as a brake upon both inflammation and lipid overload during conditions of sustained TLR ligand exposure, such as infection. miR-33 down-regula-
tion may also contribute to the late phase of Abca1 up-regulation that we observed after LPS treatment.

By reducing steady state levels of miR-33 in the macrophage, we demonstrate that native miR-33 exerts a tonic effect to enhance proinflammatory signaling by TLR4 and other TLRs. We propose that, unlike previously described miRNA regulators of TLR cascades, this occurs through an indirect mechanism, namely augmentation of cholesterol-enriched lipid raft membrane microdomains via dual suppression of ABCA1 and ABCG1. ABCA1-dependent lipid raft reduction has previously been reported to decrease proinflammatory cytokines in LPS-exposed macrophages (25, 33). The present report is, to our knowledge, the first to directly address the impact of miR-33 on TLR-induced inflammation. Our findings may, however, inform recent publications that have noted that in vivo treatment of mice with anti-miR-33 oligonucleotides reduces proinflammatory gene expression (IL-1, TNFα, and iNOS) and increases anti-inflammatory gene expression (IL-10) in atherosclerotic lesions (34–36). Our results are also consistent with a recent report demonstrating that miR-33 positively regulates proinflammatory M1 programming of macrophages by LPS/IFNγ (35).

In vivo, miR-33 may possibly regulate inflammation through additional, related mechanisms. For example, miR-33 has been shown to reduce plasma HDL via repression of hepatocyte ABCA1 (9–11, 36, 37). Given that HDL potently inhibits the innate immune response by sequestering LPS, attenuating proinflammatory cell signaling, and inhibiting myeloid cell production in the bone marrow (38, 39), miR-33 action in hepatocytes may thus also indirectly communicate via HDL regulation to impact the myeloid innate immune response. Several miRNAs are reportedly carried as cargo by plasma HDL and may thereby impact gene expression by distant cells. Through regulating HDL biogenesis, miR-33 may conceivably serve as a master regulator of endocrine delivery of other miRNAs that impact inflammation as well as other cellular processes. Lastly, given that cellular cholesterol overload can also induce inflammation through endoplasmic reticulum stress (40) and inflammasome activation (41), the potential also exists for miR-33 to impact these inflammatory mechanisms.

Several additional miRNAs have recently been identified that, like miR-33, target ABCA1 and reduce cholesterol efflux. These include miR-758 (42), miR-26 (43), miR-106b (44), miR-10b (45), miR-144 (46, 47), and miR-101 (48). We propose that studies are now warranted to address whether these microRNAs exert similar ABCA1-dependent actions on proinflammatory cytokine induction by TLRs. As these miRNAs likely have varying tissue distributions and conditions for induction/repression, it is possible that their relative impact on the innate immune response may depend on context, site, and exposure conditions. Of note, anthocyanins up-regulate ABCA1/ABCG1 in vivo via repression of miR-10b (45). Whether this mechanism contributes to the reported actions of anthocyanins to inhibit LPS-induced inflammation via ABCG1-dependent lipid raft disruption (49) is an interesting question worthy of further study.

Although we show that the miR-33 proinflammatory effect requires ABC lipid transporters and is reversible by membrane cholesterol interventions and LXR agonists, we do not discount the possibility that miR-33 may impact inflammation through additional cholesterol-independent mechanisms. For example, miR-33 was recently reported to repress the tumor suppressor p53 (50); we and others have reported that p53-deficient mice display enhanced proinflammatory gene expression in response to LPS due to derepression of NF-κB (51, 52). Alternatively, the potential for miR-33 to suppress inflammation in some contexts is also suggested by a recent report that miR-33 represses the NF-κB co-activator receptor-interacting protein-1 (53).

The degree to which our findings may be extrapolated to human cells, in which miR-33b is co-expressed with SREBP-1 and in which miR-33a/b do not repress ABCG1 (9, 10), is uncertain. miR-33b also represses ABCA1 and is thought likely to have a set of target genes very similar to those of miR-33a (9, 10). In addition to low sterols, SREBP-1 is induced by other stimuli, including insulin and LXR agonists (17). Our findings thus at least raise the interesting possibility that high insulin states in humans (e.g., metabolic syndrome) may promote inflammation in part through induction of miR-33b. They also suggest the possibility that LXR agonists, which are well-established to have anti-inflammatory actions (28), may exert some proinflammatory effects via miR-33b induction. It was recently reported that statin-induced liver injury is mediated by miR-33 regulation of biliary transporters (54). Whether statins induce liver injury as well as other toxic effects, such as myositis, in part through miR-33-promoted inflammation is a further question raised by our work.

In sum, we identify miR-33 as a TLR-responsive, indirect regulator of the innate immune response that regulates TLR induction of cytokines via ABCA1/ABCG1-mediated control of membrane microdomains. These findings identify a new fundamental node of cross-talk between lipid homeostasis and the innate immune response and may suggest new effects of, and potential applications for, therapeutic targeting of miR-33 in human disease.

**Experimental Procedures**

**Reagents**—Escherichia coli 0111:B4 LPS was purchased from List Biological Laboratories (Campbell, CA). Poly(I:C), peptidoglycan, and heat-killed L. monocytogenes were from InvivoGen (San Diego, CA). TO901317, nystatin, filipin, methyl-β-cyclodextrin, and pyrrolidine dithiocarbamate were from Sigma-Aldrich.

**Cell Culture and Transfection**—RAW 264.7 cell lines were obtained from American Type Culture Collection (ATCC) and cultured (37 °C in 5% CO2) in DMEM with 10% FCS. Thioglycollate-elicited mouse peritoneal macrophages were prepared and cultured as described previously (51). A total of 1 × 10^6 cells were seeded into each well of 24-well plates, incubated overnight, and then transfected with 25–40 nM miRNA-33 mimic or miRNA-33 inhibitor (Ambion) using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturers' instructions. Negative control mimics or negative control inhibitor were transfected as matched controls. Peritoneal macrophages were used for all murine strains with the excep-
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tion of Ch25h−/− for which bone marrow-derived macrophages were prepared using a standard protocol (55).

Mice—Female mice (8–12 weeks old, weighing 15–22 g) were used in all experiments. Myd88−/−, Traf−/−, myeloid-specific Abca1−/−, Ch25h−/−, and Abcg1−/− mice were >8–10 generations backcrossed to a C57BL/6 background as described previously (25, 56–58). Ifi191−/− mice on a C57BL/6 background and wild type C57BL/6 mice were from The Jackson Laboratory (Bar Harbor, ME). All experiments were performed in accordance with the Animal Welfare Act and the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by the Animal Care and Use Committee of the National Institute of Environmental Health Sciences.

RNA Isolation and Real Time Quantitative PCR—RNA was isolated using the mirNeasy Mini kit (Qiagen) following the manufacturer’s instructions. Real time quantitative PCR using TaqMan® Gene Expression Assays (Applied Biosystems) was performed on a 7500 Real-Time PCR System (Applied Biosystems). TaqMan microRNA assays (Applied Biosystems, Life Technologies) were used to detect and quantify mature miR-33.

ELISA and Multiplex Cytokine Assays—After stimulation with LPS at the indicated time points, cell supernatants were collected and analyzed using ELISA kits (eBioscience) or Bioplex (Bio-Rad) according to the manufacturers’ protocols.

Immunoblotting—Cells were washed twice with ice-cold PBS and lysed with cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with PMSF (Beyotime). The lysates were loaded onto SDS-polyacrylamide gels for electrophoresis and transferred onto nitrocellulose membranes as described previously (51). Detection of proteins on film was conducted and analyzed the results of experiments in the manuscript.

Flow Cytometry—To examine lipid rafts, cells were stained with 0.5 μg/ml Alexa Fluor 488-cholera toxin B (Molecular Probes) for 15 min at 4 °C. Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

FreeCholesterolQuantitation—Free cholesterol mass was measured using the Amplex® Red Cholesterol Assay kit (Thermo Fisher Scientific) according to the manufacturer’s instructions but omitting cholesterol esterase similar to a past report (59). Cholesterol was normalized to protein as measured by BCA assay (Thermo Fisher Scientific).

Statistical Analysis—All experiments were repeated at least three times. Analysis was performed using GraphPad Prism statistical software (San Diego, CA). Data are represented as mean ± S.E. Two-tailed student’s t test was applied for comparisons of two groups, and analysis of variance was applied for analyses of three or more groups. For all tests, p < 0.05 was considered significant.

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