LDL receptor-related protein-1 regulates NFκB and microRNA-155 in macrophages to control the inflammatory response

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LDL receptor-related protein-1 (LRP1) is an endocytic and cell-signaling receptor. In mice in which *LRP1* is deleted in myeloid cells, the response to lipopolysaccharide (LPS) was greatly exacerbated. *LRP1* deletion in macrophages in vitro, under the control of tamoxifen-activated Cre-ER^T fusion protein, robustly increased expression of proinflammatory cytokines and chemokines. In LRP1-expressing macrophages, proinflammatory mediator expression was regulated by LRP1 ligands in a ligand-specific manner. The LRP1 agonists, α_2-macroglobulin and tissue-type plasminogen activator, attenuated expression of inflammatory mediators, even in the presence of LPS. The antagonists, receptor-associated protein (RAP) and lactoferrin (LF), and LRP1-specific antibody had the entirely opposite effect, promoting inflammatory mediator expression and mimicking *LRP1* deletion. NFκB was rapidly activated in response to RAP and LF and responsible for the initial increase in expression of proinflammatory mediators. RAP and LF also significantly increased expression of microRNA-155 (miR-155) after a lag phase of about 4 h. miR-155 expression reflected, at least in part, activation of secondary cell-signaling pathways downstream of TNFα. Although miR-155 was not involved in the initial induction of cytokine expression in response to LRP1 antagonists, miR-155 was essential for sustaining the proinflammatory response. We conclude that LRP1, NFκB, and miR-155 function as members of a previously unidentified system that has the potential to inhibit or sustain inflammation, depending on the continuum of LRP1 ligands present in the macrophage microenvironment.

LDL receptor-related protein-1 | tissue-type plasminogen activator | lipopolysaccharide | NFκB | microRNA-155

Innate immunity is a phylogenetically conserved system that provides a first line of defense against pathogens (1, 2). Pattern recognition receptors (PRRs), including members of the Toll-like receptor (TLR) family, play an important role in innate immunity, binding microorganism-derived molecules, and initiating proinflammatory cell signaling (1, 3, 4). The effector systems of innate immunity, including proinflammatory cytokines and complement, may be very potent and when regulatory mechanisms fail, shock and death may result (3). Diverse diseases are exacerbated by dysregulated innate immunity, including Crohn's disease, rheumatoid arthritis, asthma, psoriasis, atherosclerosis, and cancer (3, 5, 6). Understanding mechanisms that control innate immunity is a significant problem in medicine.

LDL receptor-related protein-1 (LRP1) is an endocytic and cell-signaling receptor, which is essential for embryonic development (7, 8). In adults, there is increasing evidence that LRP1 regulates inflammation (9). LRP1-deficient macrophages, isolated from mice in which LRP1 is conditionally deleted in myeloid cells (mLRP1$^{-/-}$ mice), express increased levels of proinflammatory chemokines, including monocyte chemotactic protein/CCL2, MIP-1α/CCL3, and MIP-1β/CCL4 (10–12). These macrophages also migrate more rapidly, due to activation of CCL3-CCR5 signaling (10) and express decreased levels of biomarkers associated with M2 polarization (13).

In syngeneic tumors in mLRP1$^{-/-}$ mice, LRP1-deficient macrophages accumulate in increased number and express increased levels of CCL3 (10). Macrophage infiltration is also increased in atherosclerotic lesions in mLRP1$^{-/-}$ mice (11). However, mechanisms by which LRP1 regulates macrophage physiology remain incompletely understood. LRP1 deficiency is associated with increased NFκB activity in passaged cell lines (12); however, "loss of function" model systems do not address the role of LRP1 as a receptor for diverse ligands (7, 14). In neurons and neuron-like cells, different LRP1 ligands elicit distinct cell-signaling responses by engaging separate LRP1 coreceptors (15–17). If LRP1 regulates macrophage physiology in a ligand-specific manner, this would represent a powerful mechanism by which macrophages may respond to changes in their microenvironment.

In this study, we challenged mLRP1$^{-/-}$ mice and control mLRP1$^{+/+}$ mice with lipopolysaccharide (LPS), which is a major ligand for the PRR, TLR4 (18). The response to LPS was greatly exacerbated in mLRP1$^{-/-}$ mice. Using a second genetic model system, we confirmed that *LRP1* gene deletion in macrophages increases expression of proinflammatory mediators. We then showed that expression of proinflammatory mediators is controlled in macrophages by LRP1 ligands in a ligand-specific

Significance

We have identified the transmembrane receptor, LDL receptor-related protein-1 (LRP1), as a regulator of innate immunity and inflammatory responses in macrophages. *LRP1* gene deletion in myeloid cells is proinflammatory in mice, as is *LRP1* gene deletion in cultured macrophages. In LRP1-expressing macrophages, different LRP1 ligands have opposing effects on LRP1 activity, promoting or attenuating inflammatory mediator expression. NFκB is rapidly regulated by LRP1 ligands and responsible for the initial effects of LRP1 on macrophage gene expression. Regulation of microRNA-155 (miR-155) expression is a key downstream event, which may sustain or inhibit the macrophage inflammatory response. LRP1, NFκB, and miR-155 are thus members of a previously unidentified system, with the potential to control inflammation in a macrophage microenvironment-dependent manner.

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manner. LRP1 agonists, such as α_2-macroglobulin (α_2M) and tissue-type plasminogen activator (tPA), suppressed expression of proinflammatory mediators, even in the presence of LPS. Antagonists, such as receptor-associated protein (RAP) and lactoferrin (LF), increased expression of the identical mediators, mimicking the effects of *LRP1* gene deletion. The activity of LRP1 ligands was linked to regulation of NFκB and the previously unreported ability of LRP1 to control expression of microRNA-155 (miR-155) (19). LRP1, NFκB, and miR-155 emerge as members of a novel system that may control the macrophage inflammatory response in a microenvironment-sensitive manner.

Results

The Response to LPS Is Exacerbated in mLRP1$^{-/-}$ Mice. In mLRP1$^{-/-}$ mice, *LRP1* is deleted in cells in which the lysozyme-M (LysM) promoter is active, including monocytes, macrophages, neutrophils, and to some extent, dendritic cells (20). mLRP1$^{+/+}$ mice are homozygous for the floxed LRP1 gene but LysM-*Cre*-negative (21). Although LRP1 has been identified in neutrophils (22), its function in these cells is not well studied. By contrast, LRP1 is a known regulator of monocytes and macrophages (9). In the absence of experimental immune system challenge, we did not identify abnormal inflammation in 12-wk-old mLRP1$^{-/-}$ mice. TNFα and CCL3 were nearly undetectable by ELISA in plasma from mLRP1$^{-/-}$ mice. In the lungs of mLRP1$^{-/-}$ mice, myeloid cells were restricted mainly to interstitial spaces and unchanged in abundance compared with mLRP1$^{+/+}$ mice, as determined by CD11b immunohistochemistry (IHC) (Fig. 1*A*) and image analysis.

mLRP1$^{-/-}$ and mLRP1$^{+/+}$ mice were injected i.p. with LPS (n = 10–11) or vehicle. The LPS dose was set at 50% of the LD_{50} to optimize our opportunity to detect increased sensitivity. No toxicity or mortality was observed in vehicle-treated controls, as anticipated (Fig. 1*B*). One mLRP1$^{+/+}$ mouse succumbed to LPS. By contrast, 80% of the mLRP1$^{-/-}$ mice succumbed within 24 h of LPS injection. The increase in mortality of LPS-treated mLRP1$^{-/-}$ mice, compared with mLRP1$^{+/+}$ mice, was statistically significant as determined by log-rank test ($P < 0.001$).

Plasma was sampled 0, 3, 6, and 24 h after LPS injection. TNFα protein was increased in plasma from mLRP1$^{-/-}$ mice, compared with mLRP1$^{+/+}$ mice, at 3 and 6 h (Fig. 1*C*). CCL3 was also increased at 3 and 6 h (Fig. 1*D*). Quantitative reverse transcription–PCR (RT-qPCR) was performed to assess expression of proinflammatory genes in tissues harvested 24 h after injecting LPS or vehicle. IL-6 and inducible nitric oxide synthase (iNOS) mRNA were significantly increased in the lungs (Fig. 1 *E* and *F*) and kidneys (Fig. 1 *G* and *H*) of LPS-treated mLRP1$^{-/-}$ mice, compared with mLRP1$^{+/+}$ mice. In vehicle-treated mLRP1$^{-/-}$ and mLRP1$^{+/+}$ mice, IL-6 and iNOS mRNA were not significantly different, again indicating that significant inflammation is not present under basal conditions in mLRP1$^{-/-}$ mice.

Analysis of a Second Model of *LRP1* Gene Deletion in Macrophages. In mLRP1$^{-/-}$ mice, *LRP1* is deleted during development (10, 11), allowing for compensatory changes in the physiology of these cells. To address this issue, we generated homozygous LRP1$^{flox/flox}$ mice that express tamoxifen (TAM)-activated *Cre*-fusion protein (Cre-ER^T) (23). Bone-marrow–derived macrophages (BMDMs) were isolated and LRP1 protein expression was studied. Fig. 2*A* shows that, in the absence of TAM, Cre-ER^T-positive and -negative BMDMs expressed similar levels of LRP1. After treatment with 5 μM TAM for 7 d, LRP1 protein expression was neutralized in Cre-ER^T-positive cells and unchanged in Cre-ER^T-negative cells.

When *LRP1* was deleted in Cre-ER^T-positive BMDMs, expression of TNFα, IL-6, IL-1β, CCL2, and CCL3 was increased (Fig. 2 *B*–*F*). TAM did not affect cytokine expression in Cre-ER^T-negative cells. *LRP1* gene deletion also stimulated cell migration

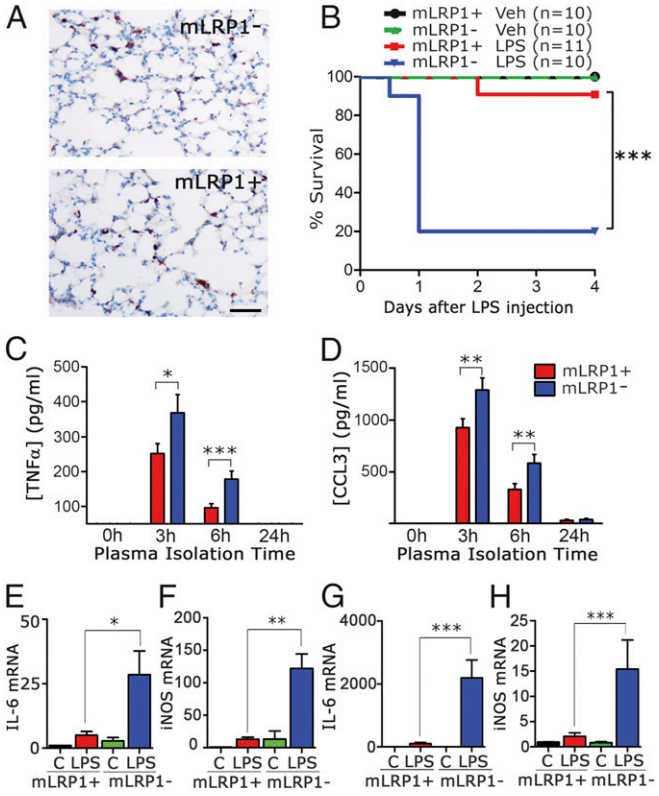

Fig. 1. LPS toxicity is increased in mice in which *LRP1* is deleted in myeloid cells. (*A*) IHC was performed to detect CD11b-positive myeloid cells in sections of lung from untreated mLRP1$^{+/+}$ and mLRP1$^{-/-}$ mice. (Scale bar, 100 μm.) Image analysis did not reveal a difference in the density of CD11b-positive cells (n = 3). (*B*) Kaplan–Meier survival curves are shown for mLRP1$^{-/-}$ and mLRP1$^{+/+}$ mice treated by i.p. injection with 1 mg/kg LPS or vehicle (Veh). Significance was determined by log-rank test (***$P < 0.001$). (*C* and *D*) ELISAs were performed to detect TNFα and CCL3 in plasma samples harvested at the indicated times from LPS-treated mLRP1$^{-/-}$ (blue bar) and mLRP1$^{+/+}$ (red bar) mice (mean ± SEM; n = 10; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; one-way ANOVA with Tukey's post hoc analysis). (*E*–*H*) RNA was harvested from the lungs (*E* and *F*) and kidneys (*G* and *H*) of mLRP1$^{+/+}$ and mLRP1$^{-/-}$ mice 24 h after i.p. injection of LPS or vehicle (C). IL-6 and iNOS mRNA were determined by RT-qPCR (n = 4).

($P < 0.001$) (Fig. 2 *G* and *H*), mimicking the results obtained with BMDMs from mLRP1$^{-/-}$ mice (10). TAM-induced *LRP1* gene deletion did not increase apoptosis, determined 8 h after transferring cells to serum-free medium (SFM), or alter the number of viable cells, determined by trypan blue exclusion at 24 h (Fig. S1).

LRP1 Ligands Regulate Macrophage Physiology in a Ligand-Specific Manner. In neurons and neuron-like cells, α_2M* and tPA function as LRP1 agonists, activating cell-signaling factors such as c-Src, ERK1/2, and Akt (15–17). LF and RAP function as antagonists, blocking cell signaling in response to agonists (16). We treated BMDMs from wild-type C57BL/6J mice with RAP (150 nM), LF (150 nM), or LPS (0.1 μg/mL) for 8 h. Fig. 3 *A*–*C* shows that RAP and LF increased expression of TNFα, IL-6, and CCL2 mRNA, similarly to LPS. RAP and LF also increased expression of IL-1β and CCL4 (Fig. S2 *A* and *B*). Fig. 3*D* shows that RAP increased CCL3 mRNA in a dose-dependent manner; 10 nM RAP was sufficient to induce a significant response. RAP and LF also increased TNFα protein levels in BMDM-conditioned medium, similarly to LPS (Fig. 3*E*).

RAP and LF stimulated BMDM cell migration (Fig. 3 *F* and *G*), mimicking the effects of *LRP1* gene deletion (10). Although inflammatory mediators are known to decrease LRP1 protein

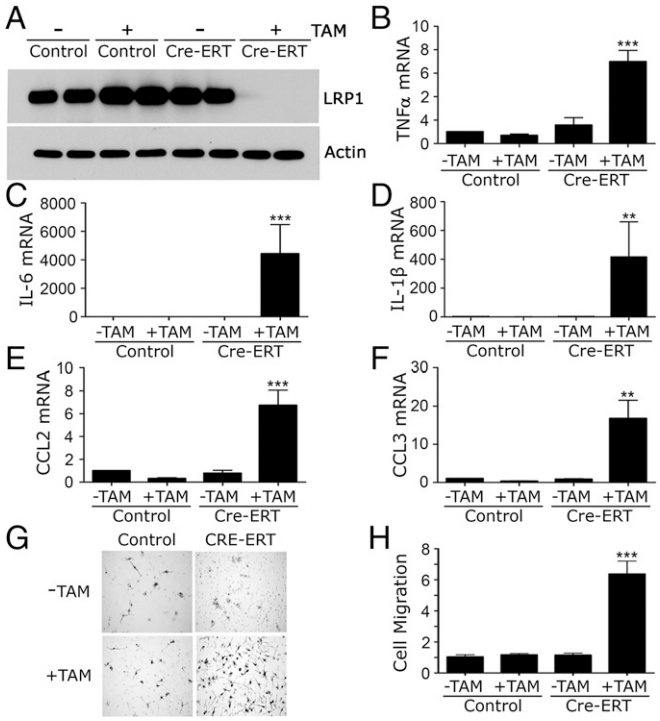

Fig. 2. *LRP1* gene deletion in macrophages in vitro induces a proinflammatory phenotype. BMDMs from Cre-ER^T-negative (control) and Cre-ER^T-positive (Cre-ERT) LRP1$^{flox/flox}$ mice were treated with 5 μM TAM (+) or vehicle (–) for 7 d. (*A*) Cell extracts were subjected to immunoblot analysis to detect the LRP1 β-chain and β-actin as a control for load. (*B–F*) RNA was isolated and RT-qPCR was performed to quantitate mRNA for (*B*) TNFα, (*C*) IL-6, (*D*) IL-1β, (*E*) CCL2, and (*F*) CCL3. (*G*) Cell migration was studied using Transwell systems. Representative images of cells that migrated to the underside surfaces of the membranes are shown. (*H*) Quantification of cell migration results (mean ± SEM; $n = 6$; $^{**}P < 0.01$, $^{***}P < 0.001$; one-way ANOVA with a Tukey's post hoc analysis).

levels in macrophages (24, 25), LRP1 protein was unchanged in BMDMs treated with LF or RAP for 8 h (Fig. 3*H*). Modest changes in LRP1 mRNA were observed in cells treated for 8 h with LF (44 ± 6%) or RAP (52 ± 5%), suggesting that longer incubations may have decreased the LRP1 protein level.

We hypothesized that RAP and LF mimic *LRP1* gene deletion by blocking LRP1 signaling initiated by agonists produced endogenously by BMDMs. LRP1 ligands are numerous and diverse (7, 9, 14). To test our hypothesis, we treated BMDMs with an antibody that binds to the LRP1 heavy chain (anti-LRP1) and blocks ligand binding. Fig. 3 *I–K* shows that anti-LRP1 induced expression of TNFα, IL-6, and CCL4, similarly to RAP and LF. In control experiments, isotype-matched IgG was without effect.

Next, we studied the LRP1 agonists: α_2M* (10 nM) and tPA (12 nM). We used an enzymatically inactive tPA variant (EI-tPA) to avoid effects unrelated to receptor-binding (26). Fig. 4*A* shows that, in wild-type BMDMs, EI-tPA and α_2M* significantly decreased TNFα expression. EI-tPA and α_2M* also decreased expression of IL-6, CCL2, and CCL3 (Fig. S3 *A–C*). Arginase-1 mRNA was increased; TGFβ mRNA was not significantly changed (Fig. S3 *D* and *E*). Because the basal level of expression of proinflammatory cytokines in BMDMs was low, we tested whether α_2M* and EI-tPA attenuate the response to LPS (0.1 μg/mL). Fig. 4 *B–G* shows that α_2M* and EI-tPA blocked the increase in expression of TNFα, IL-1β, IL-6, CCL2, CCL3, and CCL4 induced by LPS. The activity of EI-tPA was dose dependent; the minimum EI-tPA concentration that had a significant effect ($P < 0.05$) was 0.5 nM (Fig. 4*H*).

To prove that the activity of EI-tPA and α_2M* requires LRP1, we used the Cre-ER^T-LRP1$^{flox/flox}$ model system. Confirming the mechanism of EI-tPA activity was considered particularly important because tPA is an FDA-approved drug (27), known to bind to receptors in addition to LRP1 (28). TAM-treated Cre-ER^T-positive BMDMs expressed increased levels of mRNA for TNFα (Fig. 4*I*), IL-6, CCL3, and CCL4 (Fig. S3 *F–H*) as anticipated; however, when *LRP1* was deleted, EI-tPA and α_2M* failed to attenuate cytokine expression, indicating an essential role for LRP1. Similarly, in TAM-treated Cre-ER^T-LRP1$^{flox/flox}$ BMDMs, RAP and LF failed to further stimulate cytokine expression (Fig. S4), supporting the conclusion that LRP1 is the target for LF and RAP.

NFκB Functions in the Pathway by Which LRP1 Ligands Regulate Inflammation. When wild-type BMDMs were treated with RAP, IκBα was phosphorylated within 5 min (Fig. 5*A*). Total IκBα decreased substantially by 30 min, confirming NFκB activation. LF also induced IκBα phosphorylation and decreased the total abundance of IκBα (Fig. 5*B*). The effects of RAP and LF on phospho-IκBα and total IκBα were sustained for 8 h (Fig. 5*C*).

To test whether NFκB is necessary in the pathway by which LF and RAP increase cytokine expression in BMDMs, cells were treated with the NFκB nuclear translocation inhibitor, JSH-23. As a control, cells were treated with the PI3K inhibitor, LY294002. JSH-23 blocked the increase in expression of TNFα (Fig. 5*D*) and

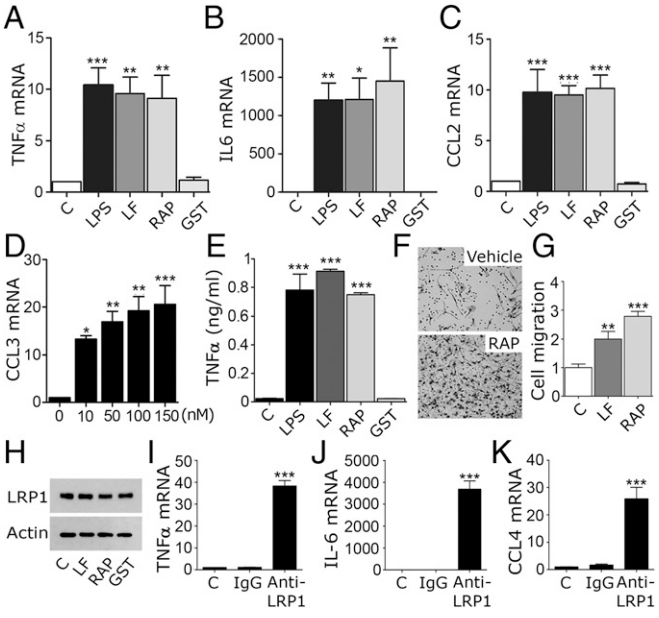

Fig. 3. LF and RAP induce expression of proinflammatory cytokines in BMDMs. (*A–C*) BMDMs from C57BL/6J mice were treated with LPS (0.1 μg/mL), LF (150 nM), RAP expressed as a GST fusion protein (150 nM), purified GST (150 nM), or vehicle (C) for 8 h. RT-qPCR was performed to determine mRNA levels for TNFα, IL-6, and CCL2. (*D*) CCL3 mRNA was determined in BMDMs treated for 8 h with increasing concentrations of RAP. (*E*) BMDMs were stimulated for 8 h with LPS, LF, RAP, GST, or vehicle as in *A*. TNFα in conditioned medium was determined by ELISA. (*F*) BMDMs were treated with LF, RAP, or vehicle (C). Cell migration was studied using Transwell systems. Representative images of migrated cells are shown. (*G*) Quantification of cell migration results ($n = 6$). (*H*) BMDMs were treated with LF, RAP, GST (each at 150 nM), or vehicle (C) for 8 h. Cell extracts were subjected to immunoblot analysis to detect the LRP1 β-chain and β-actin. (*I–K*) BMDMs were treated with LRP1-neutralizing antibody (anti-LRP1) or isotype-matched IgG for 8 h. mRNA levels were determined for TNFα, IL-6, and CCL4 (mean ± SEM; $n \geq 6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; one-way ANOVA followed by Dunnett's multiple comparison test).

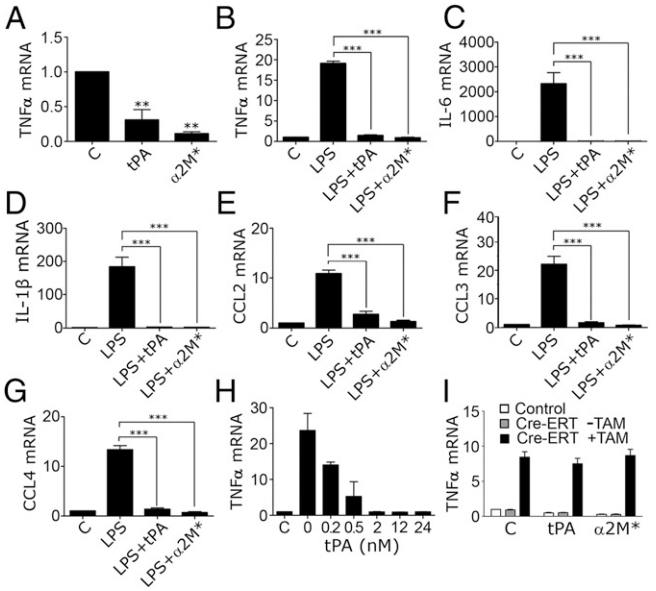

Fig. 4. α_2M* and EI-tPA inhibit expression of inflammatory mediators by BMDMs. (*A*) BMDMs from C57BL/6J mice were treated for 8 h with EI-tPA (12 nM), α_2M* (10 nM), or vehicle (C). TNFα mRNA was determined by RT-qPCR. (*B*–*G*) BMDMs were pretreated with LPS (0.1 μg/mL) for 30 min and then with EI-tPA (12 nM), α_2M* (10 nM), or vehicle (C) for 8 h. mRNA levels were determined for TNFα, IL-6, IL-1β, CCL2, CCL3, and CCL4. (*H*) BMDMs were pretreated with LPS and then with 0.2–24 nM EI-tPA for 8 h. TNFα mRNA was determined. EI-tPA concentrations of ≥0.5 nM yielded significant results ($P < 0.05$). (*I*) BMDMs from Cre-ER^T-negative (control) and Cre-ER^T-positive (Cre-ERT) LRP1$^{flox/flox}$ mice were treated with TAM (+TAM) or vehicle (−TAM) for 7 d. The Cre-ER^T-negative cells and Cre-ER^T-positive cells that were not treated with TAM both expressed LRP1. The cells were then treated with EI-tPA, α_2M*, or vehicle (C) for 8 h. TNFα mRNA was determined (mean ± SEM; $n = 4$; $^{**}P < 0.01$, $^{***}P < 0.001$; one-way ANOVA with Dunnett's or Tukey's post hoc analysis).

CCL3 (Fig. 5*E*) caused by LF and RAP. LY294002 was without effect. Fig. S5 shows that JSH-23 also blocked expression of IL-6, IL-1β, CCL2, and CCL4.

Next, we examined the ability of LRP1 agonists to block NFκB activation in response to LPS. Fig. 5*F* shows that α_2M* and EI-tPA blocked IκBα phosphorylation and the decrease in total IκB induced by LPS treatment for 1 h. The effects of α_2M* and EI-tPA were sustained for 8 h (Fig. 5*G*).

LRP1 Regulates miR-155 Expression. MicroRNAs are regulators of inflammation in various cells, including macrophages (19, 29–31). Fig. 6*A* shows that *LRP1* gene deletion in Cre-ER^T-LRP1$^{flox/flox}$ BMDMs significantly increased miR-155, without regulating miR-124, miR-9, and miR-223. In chromatin immunoprecipitation (ChiP) assays, association of the promoter region of the miR-155 parent gene with RNA polymerase II was increased when *LRP1* was deleted (Fig. 6*B*), suggesting transcriptional activation. The miR-9 promoter was not precipitated at significant levels from LRP1-expressing or -deficient cells.

Treatment of LRP1-expressing BMDMs with LF or RAP for 8 h significantly increased miR-155 (Fig. 6*C*); however, as shown in Fig. 6*D*, there was a lag phase in this response. Whereas TNFα mRNA was elevated 1 h after adding RAP, miR-155 was not significantly increased until 4–8 h. The increase in miR-155 was inhibited by JSH-23, indicating an essential role for NFκB.

Because of the lag phase in miR-155 expression, we examined the effects of inhibiting miR-155 on cytokine expression, 1 and 8 h after adding RAP. BMDMs were transfected with 10 nM *mir*VANA RNA oligonucleotide miR-155 inhibitor, which decreased miR-155 by >70%, or with control oligonucleotide. Fig. 6*E* shows that inhibiting miR-155 had no effect on TNFα mRNA expression at 1 h. By contrast, TNFα expression was significantly decreased at 8 h. Similarly, miR-155 inhibition had no effect on expression CCL4 or IL-6 at 1 h, but substantially decreased expression of these mRNAs at 8 h (Fig. S6). These results suggest that miR-155 plays an important role in sustaining the proinflammatory response to LRP1 antagonists.

We considered the possibility that miR-155 was induced as a secondary effect, downstream of cytokines such as TNFα that were expressed at increased levels by RAP-treated BMDMs. Fig. 6*F* shows that TNFα-neutralizing antibody did not affect the rapid phosphorylation of IκB in response to RAP; however, TNFα-neutralizing antibody substantially inhibited the increase in miR-155 observed 8 h after adding RAP (Fig. 6*G*).

Finally, we examined the effects of LRP1 agonists on miR-155. Fig. 6*H* shows that EI-tPA and α_2M* blocked the increase in miR-155 induced by LPS. We conclude that LRP1 functions in a ligand-specific manner to regulate miR-155.

Discussion

Despite extensive work and the availability of multiple mouse model systems, the function of LRP1 in adult mammals remains incompletely understood (9, 32). We have demonstrated a role for macrophage LRP1 in regulating innate immunity in vivo using a model system, LPS challenge, which directly tests the response of the mouse to systemic activation of the PRR, TLR4. When *LRP1* was deleted in myeloid cells, the toxicity of LPS was exacerbated and morbidity was significantly increased. Deletion of *LRP1* in vitro in BMDMs isolated from Cre-ER^T-LRP1$^{flox/flox}$ mice substantially increased expression

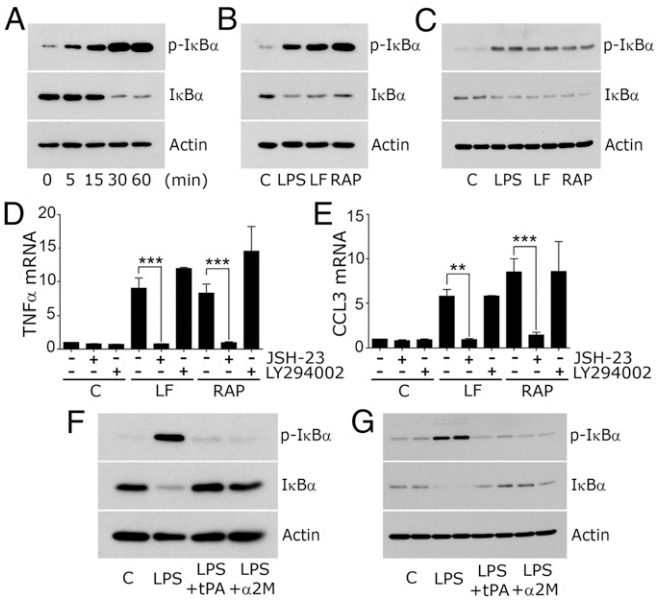

Fig. 5. LRP1 regulates NFκB activation. (*A*) BMDMs from C57BL/6J mice were treated with 150 nM RAP for the indicated times. (*B*) BMDMs were treated with vehicle (C), LPS (0.1 μg/mL), LF (150 nM), or RAP (150 nM) for 30 min. (*C*) The same incubations were conducted for 8 h. Immunoblot analysis was performed to detect phospho-IκB and total IκB. (*D* and *E*) BMDMs were pretreated with JSH-23 (10 μM), LY294002 (20 μM), or vehicle for 16 h and then with LF, RAP, or vehicle (C) for 8 h. mRNA levels were determined for TNFα and CCL3 (mean ± SEM; $n = 6$; $^{**}P < 0.01$, $^{***}P < 0.001$; one-way ANOVA with Tukey's post hoc test). (*F*) BMDMs were treated with EI-tPA (12 nM) or α_2M* (10 nM) together with LPS (0.1 μg/mL) or with LPS or vehicle (C) alone for 1 h. Immunoblot analysis was performed. (*G*) The same experiment was performed for 8 h.

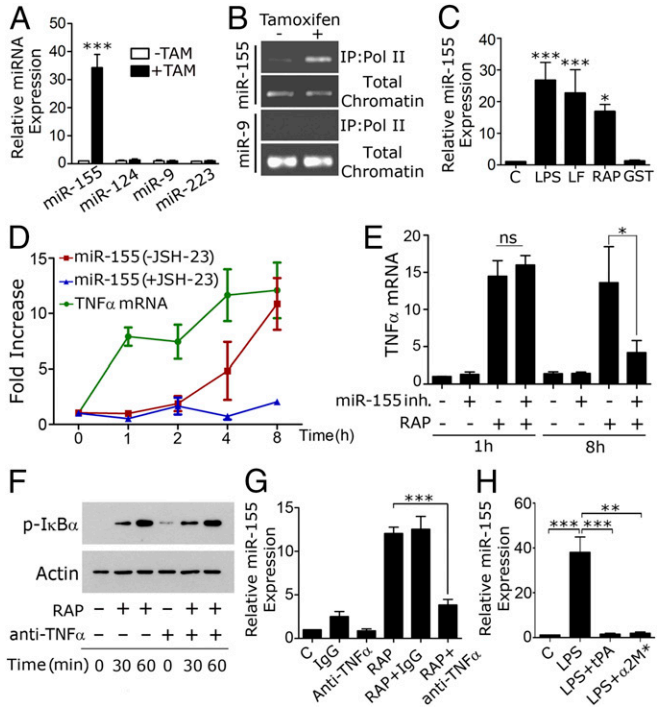

Fig. 6. miR-155 sustains the proinflammatory activity of LRP1 antagonists. (*A*) BMDMs from Cre-ER^{T}-positive-LRP1$^{flox/flox}$ mice were treated with TAM (+TAM) or vehicle (–TAM). miR-155, miR-124, miR-9, and miR-223 were determined by qPCR (mean ± SEM; $n = 5$; ***$P < 0.001$). (*B*) ChIP was performed to detect the miR-155 parent gene promoter in association with RNA polymerase II in BMDMs from Cre-ER^{T}-positive-LRP1$^{flox/flox}$ mice treated with TAM (+) or vehicle (–). The miR-9 promoter was analyzed as a control. (*C*) BMDMs from C57BL/6J mice were treated for 8 h with LPS (0.1 μg/mL), LF (150 nM), RAP (150 nM), GST (150 nM), or vehicle (C). miR-155 was determined ($n = 5$; *$P <$ 0.05, ***$P < 0.001$). (*D*) BMDMs were treated with 150 nM RAP for the indicated times. TNFα mRNA and miR-155 were determined. miR-155 also was determined in cells treated with RAP and 10 μM JSH-23 (+JSH-23). (*E*) BMDMs from C57BL/6J mice were transfected with 10 nM miR-155 inhibitor (Inh) (+) or with miRNA inhibitor negative control (–). After 48 h, the cells were treated with 150 nM RAP (+) or vehicle (–) for 1 h or 8 h. TNFα mRNA was determined ($n = 4$, ns, not statistically significant; *$P < 0.05$). (*F*) BMDMs were pretreated with TNFα-neutralizing antibody (+) or isotype-matched IgG (–) and then with RAP for 0, 30, or 60 min. Immunoblot analysis was performed. (*G*) BMDMs were treated with RAP (150 nM) or vehicle (C) alone or in the presence of TNFα-neutralizing antibody (1 μg/mL) or isotype-matched IgG for 8 h. miR-155 was determined. (*H*) BMDMs were treated with LPS (0.1 μg/mL) alone or together with El-tPA (12 nM) or α_2M* (10 nM) for 8 h. miR-155 was determined ($n = 5$; **$P < 0.01$, ***$P < 0.001$).

of multiple proinflammatory mediators. The results obtained, using this second model of LRP1 deficiency, were consistent with those obtained using BMDMs isolated from mLRP1$^{-/-}$ mice (9–12). We conclude that *LRP1* deficiency in myeloid cells in vivo and in macrophages in vitro is proinflammatory.

In LRP1-expressing macrophages, different LRP1 ligands had opposing effects on the activity of LRP1 as a regulator of the macrophage inflammatory response. RAP and LF induced rapid NFκB activation, increasing expression of the same proinflammatory mediators that were expressed by LRP1-deficient BMDMs. EI-tPA and α_2M* had the entirely opposite effect on BMDM physiology, suppressing NFκB activation in response to LPS and decreasing expression of proinflammatory mediators. In previous studies with different cell types, RAP and LF have been shown to competitively antagonize LRP1-dependent cellular responses triggered by ligands like α_2M* or tPA (15–17, 33–35). We hypothesized that, under basal conditions, macrophages express one or more agonistic ligands that bind to LRP1 in an autocrine pathway, suppressing NFκB activation. RAP and LF would then function by inhibiting binding of these endogenously produced agonists to LRP1. In support of this hypothesis, we demonstrated that LRP1-specific antibody, which blocks ligand-binding to LRP1, induces expression of inflammatory mediators in BMDMs, similarly to LF and RAP. Because the total number of LRP1 ligands is high (7, 9, 14), identifying LRP1 agonists that are secreted by macrophages represents an important proteomics problem for future investigation.

The antiinflammatory activity of EI-tPA and α_2M* was sufficiently potent to substantially inhibit and, in some cases, entirely block cytokine expression in response to LPS. Using BMDMs from Cre-ER^{T}-LRP1$^{flox/flox}$ mice, we showed that the antiinflammatory activity of EI-tPA and α_2M* requires LRP1. We conclude that the antiinflammatory activity of LRP1 may be stimulated beyond the level observed in resting macrophages by exogenously added agonists. tPA and α_2M* emerge as potentially important antiinflammatory agents. Although α_2M is abundant in plasma, α_2M circulates almost exclusively in a "nonactivated" conformation, which does not bind to LRP1 (36). The circulating level of tPA is about 0.1–0.2 nM (37), near the activity threshold demonstrated in Fig. 4.

An important consequence of *LRP1* gene deletion in BMDMs was significant and selective up-regulation of miR-155. Increased miR-155 also was observed in RAP- and LF-treated cells. In a previous study, Baltimore and colleagues (19) applied array technology and identified miR-155 as the major microRNA up-regulated in macrophages in response to diverse proinflammatory stimuli. miR-155 controls inflammation in mouse models of atherosclerosis (38) and is up-regulated in synovial fluid from patients with rheumatoid arthritis (39). When we treated BMDMs with RAP, TNFα mRNA was increased by 1 h, whereas miR-155 was not significantly increased until 4–8 h. TNFα-neutralizing antibody significantly inhibited the increase in miR-155 observed 8 h after adding RAP. These results suggest that miR-155 expression may be increased, at least in part, as a secondary response downstream of the cytokines that are expressed at increased levels when macrophages are treated with LRP1 antagonists.

miR-155 sustained the proinflammatory activity of LRP1 antagonists. Inhibiting miR-155 failed to attenuate the significant increase in TNFα expression observed 1 h after adding RAP. By contrast, 8 h after adding RAP, miR-155 inhibition substantially decreased expression of TNFα, CCL4, and IL-6. These results suggest that proinflammatory LRP1 ligands may trigger a positive feedback loop by increasing expression of cytokines such as TNFα, which induce expression of miR-155. In turn, miR-155 promotes expression of proinflammatory mediators such as TNFα. This type of positive feedback loop may be important in chronic inflammation.

JSH-23 blocked the increase in miR-155 induced by RAP in BMDMs, consistent with previous studies demonstrating that NFκB activation promotes miR-155 expression (40, 41). In our model system, NFκB inhibition may have inhibited transcription of the miR-155 parent gene directly downstream of LRP1, or expression of the cytokines that increase miR-155, or the cell-signaling pathways activated by these cytokines. In any case, LRP1, NFκB, and miR-155 emerge as members of a system that may be modulated to either promote or inhibit macrophage inflammatory responses, depending on the continuum of LRP1 ligands present in the macrophage microenvironment.

The pathway by which LRP1 ligands regulate NFκB remains to be completely elucidated. Although we previously demonstrated that TNF receptor-1 (TNFR1) is increased in LRP1-deficient fibroblasts (12, 42), TNFR1 regulation does not provide a straightforward explanation for the activity of LRP1 ligands. LRP1 demonstrates other important activities in macrophages that may be critical in tissue injury and the inflammatory response, including its function in phagocytosis of large particles (12),

efferocytosis (15, 43), and in regulating transforming growth factor-β (44). LRP1 also is involved in antigen presentation in support of adaptive immunity (45, 46). Understanding the integrated activity of macrophage LRP1 in the regulation of injury and inflammation remains an important future goal.

Materials and Methods

Proteins and Reagents. α_2M was purified from plasma (47) and activated for binding to LRP1 (α_2M*) by reaction with 200 mM methylamine-HCl. α_2M modification was confirmed by nondenaturing PAGE (36). EI-tPA was from Molecular Innovations.

Mouse Model Systems. LRP1$^{flox/flox}$ mice were bred with mice that express *Cre* recombinase under the control of the lysozyme-M promoter (LysM-*Cre*), in the C57BL/6J background, to generate mLRP1$^{-/-}$ mice. Littermate controls (mLRP1$^{+/+}$) were LRP1$^{flox/flox}$ and LysM-*Cre*-negative. LRP1$^{flox/flox}$ mice also were bred with CreER^{T} mice (23), which express Tam-activated *Cre* in all cells (Jackson Laboratories). BMDMs were isolated from littermates that were LRP1$^{flox/flox}$ and either Cre-ER^{T}-positive or -negative. Littermates were born at a 50:50 ratio. All experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee.

For additional details on proteins and reagents, LPS challenge studies, analyzing BMDM responses to LRP1 ligands, immunoblot analysis, miRNA extraction and RT-PCR, gene expression analysis, Transwell cell migration assays, ChIP assays, miRNA inhibitor studies, and statistical methods, please see *SI Materials and Methods*.

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1. Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. *Cell* 124(4):783–801.
2. Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA (1999) Phylogenetic perspectives in innate immunity. *Science* 284(5418):1313–1318.
3. Takeuchi O, Akira S (2010) Pattern recognition receptors and inflammation. *Cell* 140(6):805–820.
4. Loo YM, Gale M, Jr (2011) Immune signaling by RIG-I-like receptors. *Immunity* 34(5): 680–692.
5. Karin M, Lawrence T, Nizet V (2006) Innate immunity gone awry: Linking microbial infections to chronic inflammation and cancer. *Cell* 124(4):823–835.
6. Medzhitov R, Janeway C, Jr (2000) Innate immunity. *N Engl J Med* 343(5):338–344.
7. Strickland DK, Gonias SL, Argraves WS (2002) Diverse roles for the LDL receptor family. *Trends Endocrinol Metab* 13(2):66–74.
8. Herz J, Clouthier DE, Hammer RE (1992) LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. *Cell* 71(3): 411–421.
9. Gonias SL, Campana WM (2014) LDL receptor-related protein-1: A regulator of inflammation in atherosclerosis, cancer, and injury to the nervous system. *Am J Pathol* 184(1):18–27.
10. Staudt ND, et al. (2013) Myeloid cell receptor LRP1/CD91 regulates monocyte recruitment and angiogenesis in tumors. *Cancer Res* 73(13):3902–3912.
11. Overton CD, Yancey PG, Major AS, Linton MF, Fazio S (2007) Deletion of macrophage LDL receptor-related protein increases atherogenesis in the mouse. *Circ Res* 100(5): 670–677.
12. Gaultier A, et al. (2008) Regulation of tumor necrosis factor receptor-1 and the IKK-NF-kappaB pathway by LDL receptor-related protein explains the antiinflammatory activity of this receptor. *Blood* 111(11):5316–5325.
13. May P, Bock HH, Nofer J-R (2013) Low density receptor-related protein 1 (LRP1) promotes anti-inflammatory phenotype in murine macrophages. *Cell Tissue Res* 354(3):887–889.
14. Fernandez-Castaneda A, et al. (2013) Identification of the low density lipoprotein (LDL) receptor-related protein-1 interactome in central nervous system myelin suggests a role in the clearance of necrotic cell debris. *J Biol Chem* 288(7):4538–4548.
15. Stiles TL, et al. (2013) LDL receptor-related protein-1 is a sialic-acid-independent receptor for myelin-associated glycoprotein that functions in neurite outgrowth inhibition by MAG and CNS myelin. *J Cell Sci* 126(Pt 1):209–220.
16. Mantuano E, Lam MS, Gonias SL (2013) LRP1 assembles unique co-receptor systems to initiate cell signaling in response to tissue-type plasminogen activator and myelin-associated glycoprotein. *J Biol Chem* 288(47):34009–34018.
17. Shi Y, Mantuano E, Inoue G, Campana WM, Gonias SL (2009) Ligand binding to LRP1 transactivates Trk receptors by a Src family kinase-dependent pathway. *Sci Signal* 2(68):ra18.
18. Raetz CRH, Whitfield C (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* 71: 635–700.
19. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D (2007) MicroRNA-155 is induced during the macrophage inflammatory response. *Proc Natl Acad Sci USA* 104(5):1604–1609.
20. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Förster I (1999) Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res* 8(4): 265–277.
21. Rohlmann A, Gotthardt M, Hammer RE, Herz J (1998) Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. *J Clin Invest* 101(3):689–695.
22. Jardí M, et al. (1996) Distinct patterns of urokinase receptor (uPAR) expression by leukemic cells and peripheral blood cells. *Thromb Haemost* 76(6):1009–1019.
23. Hayashi S, McMahon AP (2002) Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: A tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol* 244(2):305–318.
24. LaMarre J, Wolf BB, Kittler EL, Quesenberry PJGS, Gonias SL (1993) Regulation of macrophage alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein by lipopolysaccharide and interferon-gamma. *J Clin Invest* 91(3):1219–1224.
25. Gorovoy M, Gaultier A, Campana WM, Firestein GS, Gonias SL (2010) Inflammatory mediators promote production of shed LRP1/CD91, which regulates cell signaling and cytokine expression by macrophages. *J Leukoc Biol* 88(4):769–778.
26. Declerck PJ, De Mol M, Vaughan DE, Collen D (1992) Identification of a conformationally distinct form of plasminogen activator inhibitor-1, acting as a noninhibitory substrate for tissue-type plasminogen activator. *J Biol Chem* 267(17):11693–11696.
27. Wang DZ, Rose JA, Honings DS, Garwacki DJ, Milbrandt JC (2000) Treating acute stroke patients with intravenous tPA. The OSF stroke network experience. *Stroke* 31(1):77–81.
28. Flood EC, Hajjar KA (2011) The annexin A2 system and vascular homeostasis. *Vascul Pharmacol* 54(3-6):59–67.
29. Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD (2008) MicroRNAs: New regulators of immune cell development and function. *Nat Immunol* 9(8):839–845.
30. Graff JW, Dickson AM, Clay G, McCaffrey AP, Wilson ME (2012) Identifying functional microRNAs in macrophages with polarized phenotypes. *J Biol Chem* 287(26):21816–21825.
31. Liu G, Abraham E (2013) MicroRNAs in immune response and macrophage polarization. *Arterioscler Thromb Vasc Biol* 33(2):170–177.
32. Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK (2008) LDL receptor-related protein 1: Unique tissue-specific functions revealed by selective gene knockout studies. *Physiol Rev* 88(3):887–918.
33. Bonacci GR, Cáceres LC, Sánchez MC, Chiabrando GA (2007) Activated alpha(2)-macroglobulin induces cell proliferation and mitogen-activated protein kinase activation by LRP-1 in the J774 macrophage-derived cell line. *Arch Biochem Biophys* 460(1):100–106.
34. Mantuano E, et al. (2008) The hemopexin domain of matrix metalloproteinase-9 activates cell signaling and promotes migration of schwann cells by binding to low-density lipoprotein receptor-related protein. *J Neurosci* 28(45):11571–11582.
35. Sauer H, et al. (2013) α2-Macroglobulin enhances vasculogenesis/angiogenesis of mouse embryonic stem cells by stimulation of nitric oxide generation and induction of fibroblast growth factor-2 expression. *Stem Cells Dev* 22(9):1443–1454.
36. Gonias SL, Pizzo SV (1983) Chemical and structural modifications of alpha 2-macroglobulin: Effects on receptor binding and endocytosis studied in an in vivo model. *Ann N Y Acad Sci* 421(Hl 24066):457–471.
37. Thögersen AM, et al. (1998) High plasminogen activator inhibitor and tissue plasminogen activator levels in plasma precede a first acute myocardial infarction in both men and women: Evidence for the fibrinolytic system as an independent primary risk factor. *Circulation* 98(21):2241–2247.
38. Du F, et al. (2014) MicroRNA-155 deficiency results in decreased macrophage inflammation and attenuated atherogenesis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 34(4):759–767.
39. Kurowska-Stolarska M, et al. (2011) MicroRNA-155 as a proinflammatory regulator in clinical and experimental arthritis. *Proc Natl Acad Sci USA* 108(27):11193–11198.
40. Ma X, Becker Buscaglia LE, Barker JR, Li Y (2011) MicroRNAs in NF-kappaB signaling. *J Mol Cell Biol* 3(3):159–166.
41. Kluiver J, et al. (2007) Regulation of pri-microRNA BIC transcription and processing in Burkitt lymphoma. *Oncogene* 26(26):3769–3776.
42. Gonias SL, Wu L, Salicioni AM (2004) Low density lipoprotein receptor-related protein: Regulation of the plasma membrane proteome. *Thromb Haemost* 91(6): 1056–1064.
43. Subramanian M, et al. (2014) An AXL/LRP-1/RANBP9 complex mediates DC efferocytosis and antigen cross-presentation in vivo. *J Clin Invest* 124(3):1296–1308.
44. Muratoglu SC, et al. (2011) Macrophage LRP1 suppresses neo-intima formation during vascular remodeling by modulating the TGF-β signaling pathway. *PLoS One* 6(12): e28846.
45. Bowers EV, Horvath JJ, Bond JE, Cianciolo GJ, Pizzo SV (2009) Antigen delivery by alpha(2)-macroglobulin enhances the cytotoxic T lymphocyte response. *J Leukoc Biol* 86(5):1259–1268.
46. Pawaria S, Kropp LE, Binder RJ (2012) Immunotherapy of tumors with α2-macroglobulin-antigen complexes pre-formed in vivo. *PLoS One* 7(11):e50365.
47. Imber MJ, Pizzo SV (1981) Clearance and binding of two electrophoretic "fast" forms of human alpha 2-macroglobulin. *J Biol Chem* 256(15):8134–8139.
48. Herz J, Goldstein JL, Strickland DK, Ho YK, Brown MS (1991) 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *J Biol Chem* 266(31):21232–21238.
49. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162(1):156–159.