

LDL receptor-related protein-1 regulates NF κ B 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. NF κ B 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 TNF α . 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, NF κ B, 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 | NF κ B | 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 chemoattractant protein/CCL2, MIP-1 α /CCL3, and MIP-1 β /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 NF κ B 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. NF κ B 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, NF κ B, 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_2 M) 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 NF κ B and the previously unreported ability of LRP1 to control expression of microRNA-155 (miR-155) (19). LRP1, NF κ B, 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. TNF α 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. 1A) 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 LD₅₀ to optimize our opportunity to detect increased sensitivity. No toxicity or mortality was observed in vehicle-treated controls, as anticipated (Fig. 1B). 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. TNF α protein was increased in plasma from mLRP1^{-/-} mice, compared with mLRP1^{+/+} mice, at 3 and 6 h (Fig. 1C). CCL3 was also increased at 3 and 6 h (Fig. 1D). 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. 1E and F) and kidneys (Fig. 1G 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*^{flx/flx} 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. 2A shows that, in the absence of TAM, *Cre-ER*^T-positive and -negative BMDMs expressed similar levels of *LRP1*. After treatment with 5 μ M 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 TNF α , IL-6, IL-1 β , CCL2, and CCL3 was increased (Fig. 2B–F). TAM did not affect cytokine expression in *Cre-ER*^T-negative cells. *LRP1* gene deletion also stimulated cell migration

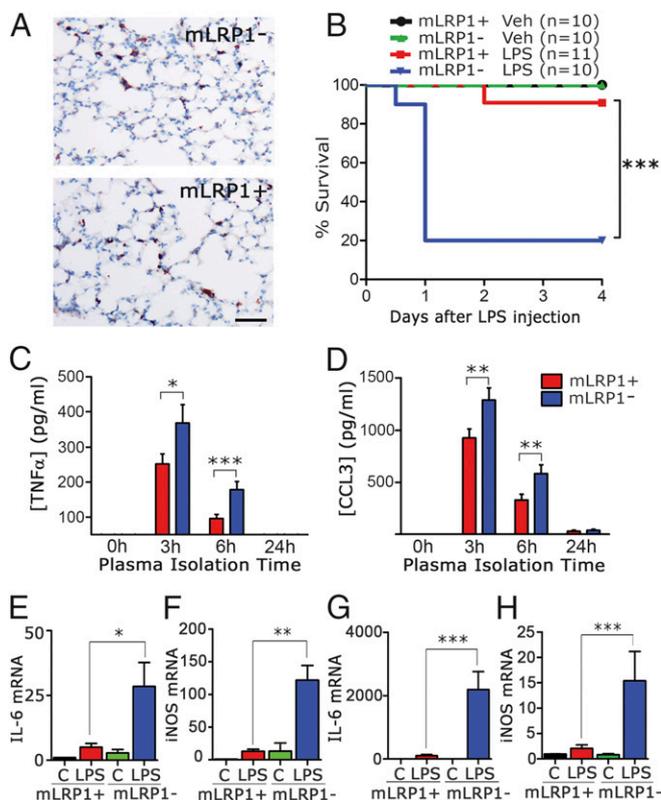


Fig. 1. LPS toxicity is increased in mice in which *LRP1* is deleted in myeloid cells. (A) IHC was performed to detect CD11b-positive myeloid cells in sections of lung from untreated mLRP1^{+/+} and mLRP1^{-/-} mice. (Scale bar, 100 μ m.) Image analysis did not reveal a difference in the density of CD11b-positive 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 TNF α and CCL3 in plasma samples harvested at the indicated times from LPS-treated mLRP1^{-/-} (blue bar) and mLRP1^{+/+} (red bar) mice (mean \pm 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. 2G 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_2 M* 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 μ g/mL) for 8 h. Fig. 3A–C shows that RAP and LF increased expression of TNF α , IL-6, and CCL2 mRNA, similarly to LPS. RAP and LF also increased expression of IL-1 β and CCL4 (Fig. S2A and B). Fig. 3D 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 TNF α protein levels in BMDM-conditioned medium, similarly to LPS (Fig. 3E).

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

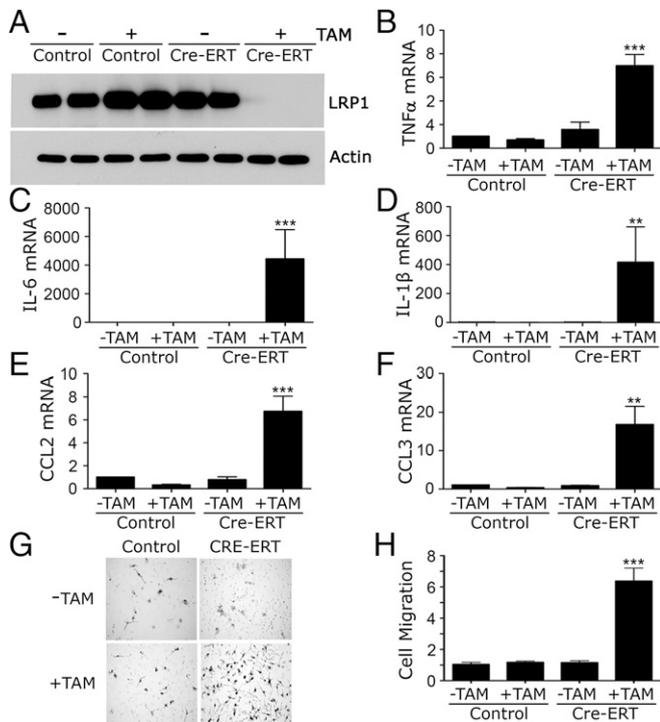


Fig. 2. *LRP1* gene deletion in macrophages in vitro induces a pro-inflammatory phenotype. BMDMs from Cre-ER^T-negative (control) and Cre-ER^T-positive (Cre-ERT) *LRP1*^{fllox/fllox} mice were treated with 5 μ M TAM (+) or vehicle (-) for 7 d. (A) Cell extracts were subjected to immunoblot analysis to 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) TNF α , (C) IL-6, (D) IL-1 β , (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 \pm 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. 3H). Modest changes in *LRP1* mRNA were observed in cells treated for 8 h with LF (44 \pm 6%) or RAP (52 \pm 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 TNF α , 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_2 M* (10 nM) and tPA (12 nM). We used an enzymatically inactive tPA variant (EI-tPA) to avoid effects unrelated to receptor-binding (26). Fig. 4A shows that, in wild-type BMDMs, EI-tPA and α_2 M* significantly decreased TNF α expression. EI-tPA and α_2 M* also decreased expression of IL-6, CCL2, and CCL3 (Fig. S3 A–C). Arginase-1 mRNA was increased; TGF β 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_2 M* and EI-tPA attenuate the response to LPS (0.1 μ g/mL). Fig. 4 B–G shows that α_2 M* and EI-tPA blocked the increase in expression of TNF α , IL-1 β , 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. 4H).

To prove that the activity of EI-tPA and α_2 M* requires *LRP1*, we used the Cre-ER^T-*LRP1*^{fllox/fllox} 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 TNF α (Fig. 4I), IL-6, CCL3, and CCL4 (Fig. S3 F–H) as anticipated; however, when *LRP1* was deleted, EI-tPA and α_2 M* failed to attenuate cytokine expression, indicating an essential role for *LRP1*. Similarly, in TAM-treated Cre-ER^T-*LRP1*^{fllox/fllox} BMDMs, RAP and LF failed to further stimulate cytokine expression (Fig. S4), supporting the conclusion that *LRP1* is the target for LF and RAP.

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

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

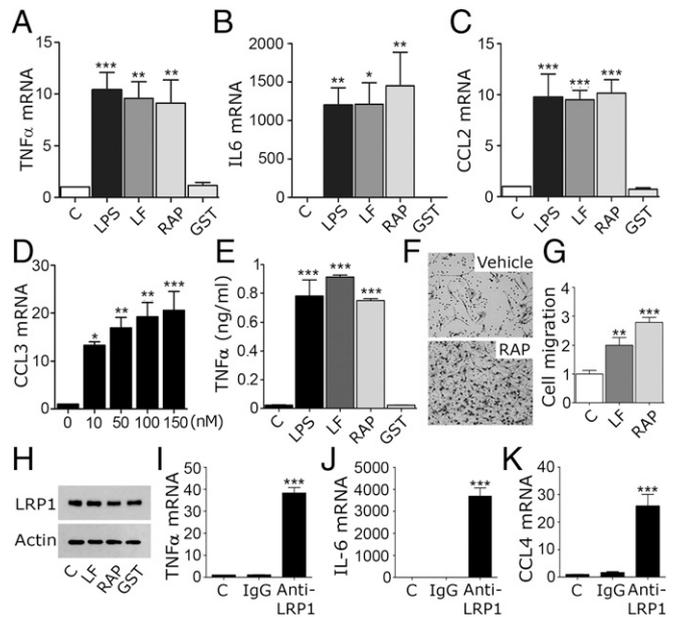


Fig. 3. LF and RAP induce expression of proinflammatory cytokines in BMDMs. (A–C) BMDMs from C57BL/6J mice were treated with LPS (0.1 μ g/mL), LF (150 nM), RAP expressed as a GST fusion protein (150 nM), purified GST (150 nM), or vehicle (C) for 8 h. RT-qPCR was performed to determine mRNA levels for TNF α , 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. TNF α 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 TNF α , IL-6, and CCL4 (mean \pm SEM; $n \geq 6$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; one-way ANOVA followed by Dunnett's multiple comparison test).

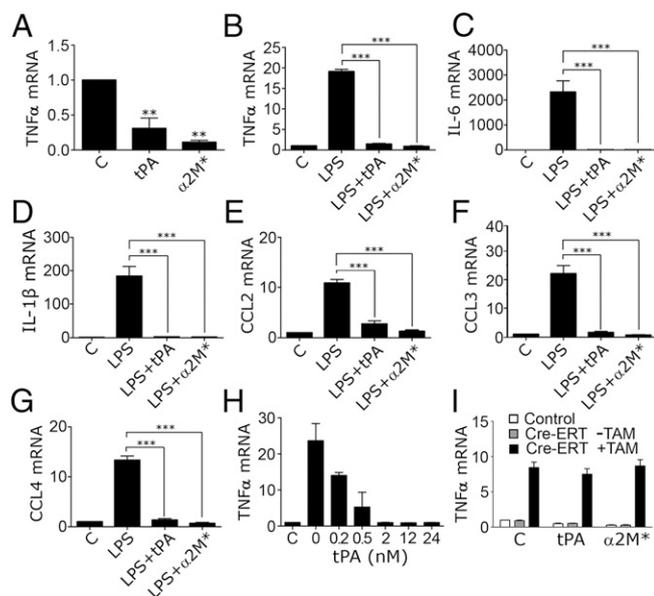


Fig. 4. α_2M^* and EI-tPA inhibit expression of inflammatory mediators by BMDMs. (A) BMDMs from C57BL/6J mice were treated for 8 h with EI-tPA (12 nM), α_2M^* (10 nM), or vehicle (C). TNF α mRNA was determined by RT-qPCR. (B–G) BMDMs were pretreated with LPS (0.1 μ g/mL) for 30 min and then with EI-tPA (12 nM), α_2M^* (10 nM), or vehicle (C) for 8 h. mRNA levels were determined for TNF α , IL-6, IL-1 β , CCL2, CCL3, and CCL4. (H) BMDMs were pretreated with LPS and then with 0.2–24 nM EI-tPA for 8 h. TNF α mRNA was determined. EI-tPA concentrations of ≥ 0.5 nM yielded significant results ($P < 0.05$). (I) BMDMs from Cre-ERT-negative (control) and Cre-ERT-positive (Cre-ERT) LRP1^{flox/flox} mice were treated with TAM (+TAM) or vehicle (–TAM) for 7 d. The Cre-ERT-negative cells and Cre-ERT-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. TNF α mRNA was determined (mean \pm SEM; $n = 4$; $**P < 0.01$, $***P < 0.001$; one-way ANOVA with Dunnett's or Tukey's post hoc analysis).

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

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

LRP1 Regulates miR-155 Expression. MicroRNAs are regulators of inflammation in various cells, including macrophages (19, 29–31). Fig. 6A shows that LRP1 gene deletion in Cre-ERT⁺-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. 6B), 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. 6C); however, as shown in Fig. 6D, there was a lag phase in this response. Whereas TNF α 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 NF κ B.

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 *mirVANA* RNA oligonucleotide miR-155 inhibitor, which decreased miR-155

by $>70\%$, or with control oligonucleotide. Fig. 6E shows that inhibiting miR-155 had no effect on TNF α mRNA expression at 1 h. By contrast, TNF α 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 TNF α that were expressed at increased levels by RAP-treated BMDMs. Fig. 6F shows that TNF α -neutralizing antibody did not affect the rapid phosphorylation of I κ B in response to RAP; however, TNF α -neutralizing antibody substantially inhibited the increase in miR-155 observed 8 h after adding RAP (Fig. 6G).

Finally, we examined the effects of LRP1 agonists on miR-155. Fig. 6H 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-ERT⁺-LRP1^{flox/flox} mice substantially increased expression

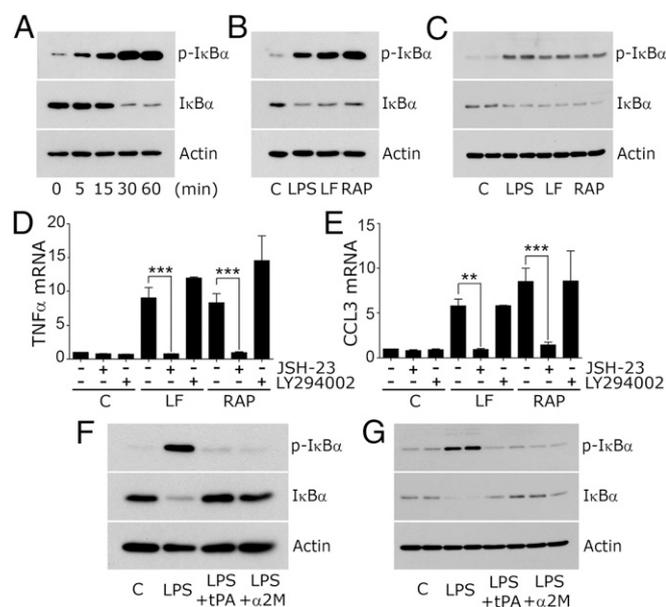


Fig. 5. LRP1 regulates NF κ B activation. (A) BMDMs from C57BL/6J mice were treated with 150 nM RAP for the indicated times. (B) BMDMs were treated with vehicle (C), LPS (0.1 μ g/mL), LF (150 nM), or RAP (150 nM) for 30 min. (C) The same incubations were conducted for 8 h. Immunoblot analysis was performed to detect phospho-I κ B and total I κ B. (D and E) BMDMs were pretreated with JSH-23 (10 μ M), LY294002 (20 μ M), or vehicle for 16 h and then with LF, RAP, or vehicle (C) for 8 h. mRNA levels were determined for TNF α and CCL3 (mean \pm 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 μ g/mL) or with LPS or vehicle (C) alone for 1 h. Immunoblot analysis was performed. (G) The same experiment was performed for 8 h.

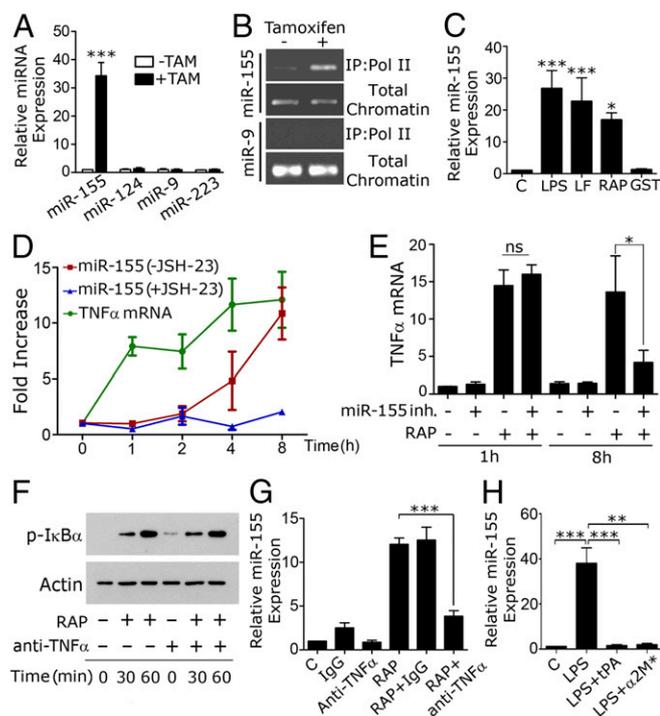


Fig. 6. miR-155 sustains the proinflammatory activity of LRP1 antagonists. (A) BMDMs from Cre-ER^T-positive-LRP1^{fllox/fllox} mice were treated with TAM (+TAM) or vehicle (–TAM). miR-155, miR-124, miR-9, and miR-223 were determined by qPCR (mean ± SEM; *n* = 5; ****P* < 0.001). (B) ChIP was performed to detect the miR-155 parent gene promoter in association with RNA polymerase II in BMDMs from Cre-ER^T-positive-LRP1^{fllox/fllox} 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 EI-tPA (12 nM) or α₂M* (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 α₂M* 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 α₂M* 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 α₂M* 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^{fllox/fllox} mice, we showed that the antiinflammatory activity of EI-tPA and α₂M* 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 α₂M* emerge as potentially important antiinflammatory agents. Although α₂M is abundant in plasma, α₂M 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_2 M was purified from plasma (47) and activated for binding to LRP1 (α_2 M*) by reaction with 200 mM methylamine-HCl. α_2 M modification was confirmed by nondenaturing PAGE (36). EI-tPA was from Molecular Innovations.

Mouse Model Systems. LRP1^{fllox/fllox} 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^{fllox/fllox} and LysM-Cre-negative. LRP1^{fllox/fllox} 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^{fllox/fllox} 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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Supporting Information

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SI Materials and Methods

Proteins and Reagents. LF and *Escherichia coli* LPS, serotype 055:B5, were from Sigma-Aldrich. RAP was expressed as a GST-fusion protein and purified as previously described (48). Free GST was expressed using empty vector. GST-fusion proteins were subjected to chromatography on Detoxi-Gel endotoxin-removing columns (Pierce). The compound 4-hydroxytamoxifen (TAM) was from Enzo Life Sciences. JSH-23 and LY294002 were from Calbiochem. Rabbit TNF α -neutralizing antibody and anti-CD11b were purchased from Abcam. Specific antibodies directed against the C-terminal 85-kDa and the N-terminal 515-kDa subunits of LRP1 and β -actin were from Sigma. Antibodies that detect phosphorylated I κ B α and total I κ B α were from Cell Signaling Technologies.

LPS Challenge. mLRP1^{-/-} and mLRP1^{+/+} mice were treated by i.p. injection with LPS (1 mg/kg). Control mice received an equivalent volume of normal saline. The mice were observed for changes in neurologic status, altered respiratory rate, and/or hemostasis abnormalities. Those entering a moribund condition were killed immediately by cervical dislocation under anesthesia. Kaplan–Meier survival curves were analyzed by log-rank test (Prism). In separate experiments, blood samples were recovered from the retroorbital venous plexus. Plasma was isolated and TNF α and CCL3 were measured using the mouse TNF α and CCL3/MIP-1 α Quantikine ELISA kits (R&D Systems). Lungs and kidneys were recovered 24 h after LPS injection. RNA was isolated and RT-qPCR was performed.

Immunohistochemistry. Lung tissue was harvested from mice, formalin fixed, processed, and paraffin embedded. Four-micrometer-thick tissue sections were cut and mounted on positively charged microscope slides. Sections were stained with antibody against CD11b (1:1,200) from Abcam using a Ventana Discovery Ultra (Ventana Medical Systems). Antigen retrieval was performed using CC1 for 40 min at 95 °C. The primary antibody was incubated on the sections for 1 h at 37 °C and visualized using 3,3'-diaminobenzidine as a chromagen and the UltraMap System (Ventana Medical Systems). Slides were counterstained with hematoxylin, rinsed, dehydrated through alcohol and xylene, and cover slipped.

Isolation of BMDMs. The 12- to 16-wk-old mice were killed. Femurs and tibiae were recovered from both rear legs. Bone marrow cells were flushed from the long bones in DMEM (Invitrogen). Cell suspensions were filtered through a 100- μ m nylon cell strainer and plated in 100-mm dishes at a density of 2×10^6 cells per dish in DMEM containing 10% (vol/vol) FBS (HyClone Laboratories) and 20% (vol/vol) L929 cell-conditioned medium. After 10 d, nonadherent cells were removed. Adherent cells, which constituted a nearly pure population of BMDMs (2, 3), were used in experiments.

BMDM Response to LRP1 Ligands. BMDMs from LRP1^{flox/flox}-Cre-ER^T-positive mice and LRP1^{flox/flox}-Cre-ER^T-negative mice were pretreated with TAM (5 μ M) or with vehicle for 7 d. These cells and cells from wild-type C57BL/6J mice were transferred to SFM for 30 min and then treated for 8 h with α_2 M*, EI-tPA, LF, or RAP. Cells were treated with LPS (0.1 μ g/mL) as a control. Treatments with vehicle or GST served as negative controls. In another set of experiments, BMDMs from wild-type C57BL/6J

mice were transferred to SFM for 30 min and treated with rabbit IgG1 polyclonal antibody directed against LRP1 heavy chain.

Immunoblot Analysis. Cellular extracts were prepared in RIPA buffer (PBS with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing Complete Protease Inhibitor Mixture (Roche Diagnostics) and sodium orthovanadate. The protein concentration in tissue extracts was determined by bicinchoninic acid (BCA) assay. An equivalent amount of cellular protein (50 μ g per lane) was subjected to SDS/PAGE and electrotransferred to methanol-activated PVDF membranes. Membranes were blocked with 5% (wt/vol) nonfat dry milk in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, with 0.1% Tween 20 and subsequently incubated with primary polyclonal antibody that recognizes the C-terminal 85-kDa subunit of LRP1 (1:1,000; Sigma); phosphorylated I κ B (1:1,000; Cell Signaling Technologies); total I κ B (1:1,000; Cell Signaling Technologies); or monoclonal antibody that detects actin (1:1,000; Sigma). Primary antibody binding was detected by HRP-conjugated species-specific secondary antibodies (1:2,000; Cell Signaling Technologies). Immunoblots were developed using enhanced chemiluminescence ECL Plus (GE Healthcare) and HyBlot CL Autoradiography film (Denville Scientific).

ELISAs. TNF α protein in conditioned medium was determined using the mouse TNF α Quantikine ELISA kit (R&D Systems). Apoptotic cell death was assessed using the Cell Death Detection Plus ELISA kit (Roche Diagnostics), which quantifies apoptotic cells by detecting histone-associated DNA fragments (mono- and oligonucleosomes).

Gene Expression Analysis. Total RNA was isolated from 40- μ m-thick sections of mouse kidneys and lungs and from BMDMs using the acid guanidinium-thiocyanate-phenol-chloroform extraction method (49) and TRIzol Reagent (Life Technologies). RNA was purified using the NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's instructions. cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad). RT-qPCR was performed using TaqMan gene expression assay products on an AB Step One Plus Real-Time PCR system (Applied Biosystems). The relative change in gene expression was calculated using the $2\Delta\Delta$ Ct method and GAPDH gene as a standardizer. Primer-probe sets were as follows: GAPDH (Mm99999915_g1); TNF α (Mm00443258_m1); IL-6 (Mm00446190_m1); IL1- β (Mm00434228_m1); CCL2 (Mm00441242_m1); CCL3 (Mm00441259_g1); and CCL4 (Mm00443111_m1).

miRNA Detection. miRNA was isolated using the mirVANA miRNA isolation kit (Applied Biosystems). miRNA (10 ng) was retrotranscribed into cDNA using the Taqman miRNA Reverse transcription kit (Applied Biosystems) and RT primers specific for: miR-155, miR-9, miR-223, and miR-124. qPCR was performed in duplicate using Taqman Universal PCR Master Mix, No AmpErase UNG, and the following miRNA-specific probe mixes (Applied Biosystems): mmu-miR-155 (002571); mmu-miR-223-5 (007896_mat); hsa-miR-9 (000583); and mmu-miR-124a (001182). The relative change in gene expression was calculated using the $2\Delta\Delta$ Ct method and *snoRNA202* (001232) as a control.

miRNA Inhibitor Studies. BMDMs isolated from C57BL/6J mice were transfected with 10 nM miR-155 inhibitor (mmu-miR-155-5p, Applied Biosystems) using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's

instructions. As positive and negative controls, cells were transfected with the mirVana miRNA Inhibitor let-7c Positive Control and mirVana miRNA Inhibitor Negative Control 1 (Ambion). miR-155 expression was determined by qPCR to confirm depletion. Subsequently, BMDMs were treated with LF or RAP, beginning 48 h after transfection with miR-155 inhibitor.

Transwell Cell Migration Assays. BMDMs (5×10^5) were added to the top chamber of 24-well Transwell units with 8.0- μm pores. The underside of each membrane was coated with 10 $\mu\text{g}/\text{mL}$ fibronectin (Millipore). When BMDMs were pretreated with LRP1 ligands, including RAP (150 nM) or LF (150 nM), the same ligands were added to the medium in the Transwells. The bottom chamber contained 10% (vol/vol) FBS. Cells were allowed to migrate for 16 h. Nonmigrating cells were removed from the upper surface. The lower surface was stained with Hema 3 (Fisher Scientific). Stained membranes were imaged using a Leica DMIRE2 microscope. The number of migrated cells was determined in four representative fields, selected by a blinded investigator, using ImageJ software. Three separate membranes were analyzed for each condition in three separate experiments.

ChIP. BMDMs (1×10^7) were treated with 0.75% formaldehyde. Cross-linking was terminated with 0.12 M glycine. Nuclei were

isolated from cell extracts by centrifugation at $600 \times g$. Chromatin was sheared into 200- to 1,000-bp fragments by sonication. Cell debris was cleared by centrifugation and the chromatin filtered using a 0.2- μm filter. For immunoprecipitation (IP), sheared chromatin was incubated with anti-RNapol II antibody (Abcam), which recognizes the phospho-S-5 epitope in the C-terminal domain of activated RNA polymerase II, for 2 h at 4 °C and Protein G-Sepharose. DNA was eluted in 1% SDS and treated with RNase A and Proteinase K. DNA was purified using the minElute gel extraction kit (Qiagen). PCR was performed using Phusion polymerase (NEB). The primers (5'-GTTTCCTTTAAGAA-TAAAGCA-3' and 5'-GTTCTTGAACCTCCGGC-TCGA-3') amplified a region near the TATA box of the *BIC* gene, which encodes miR-155.

Statistics. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software). All results are expressed as mean \pm SEM. Survival studies were analyzed by log-rank test. ELISA results, qPCR data, and cell migration studies were analyzed by one-way ANOVA followed by Tukey's or Dunnett's post hoc analysis. Differences between two experimental groups were evaluated using a Mann-Whitney test. In the SI figures, statistical significance is indicated as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

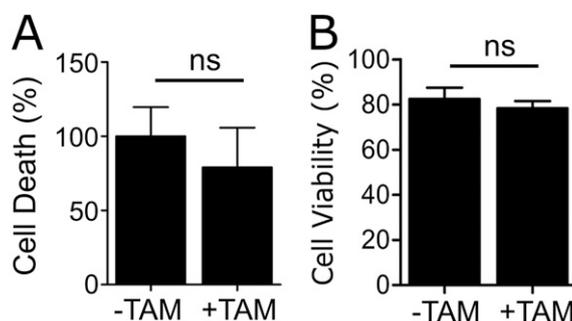


Fig. S1. *LRP1* gene deletion does not compromise cell viability. BMDMs were isolated from Cre-ER^T-positive-*LRP1*^{fllox/fllox} mice and treated with 5 μM TAM (+TAM) or vehicle (–TAM). (A) Apoptosis was measured using the Cell Death ELISA. (B) Cell viability was determined by trypan blue exclusion (mean \pm SEM; $n = 4$; ns, not statistically significant; Mann-Whitney test).

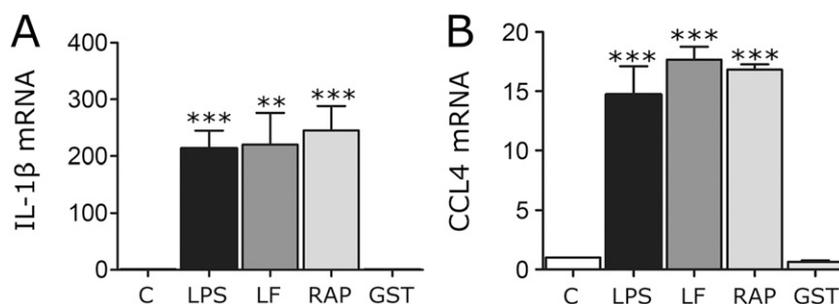


Fig. S2. LF and RAP increase expression of proinflammatory mediators. BMDMs from C57BL/6J mice were treated with LPS (0.1 $\mu\text{g}/\text{mL}$), LF (150 nM), RAP, which was expressed as a GST-fusion protein (150 nM), purified GST (150 nM), or vehicle (C) for 8 h. RT-qPCR was performed to quantify mRNA levels for (A) IL-1 β and (B) CCL4 (mean \pm SEM; $n = 6$; ** $P < 0.01$, *** $P < 0.001$; one-way ANOVA followed by Dunnett's post hoc analysis).

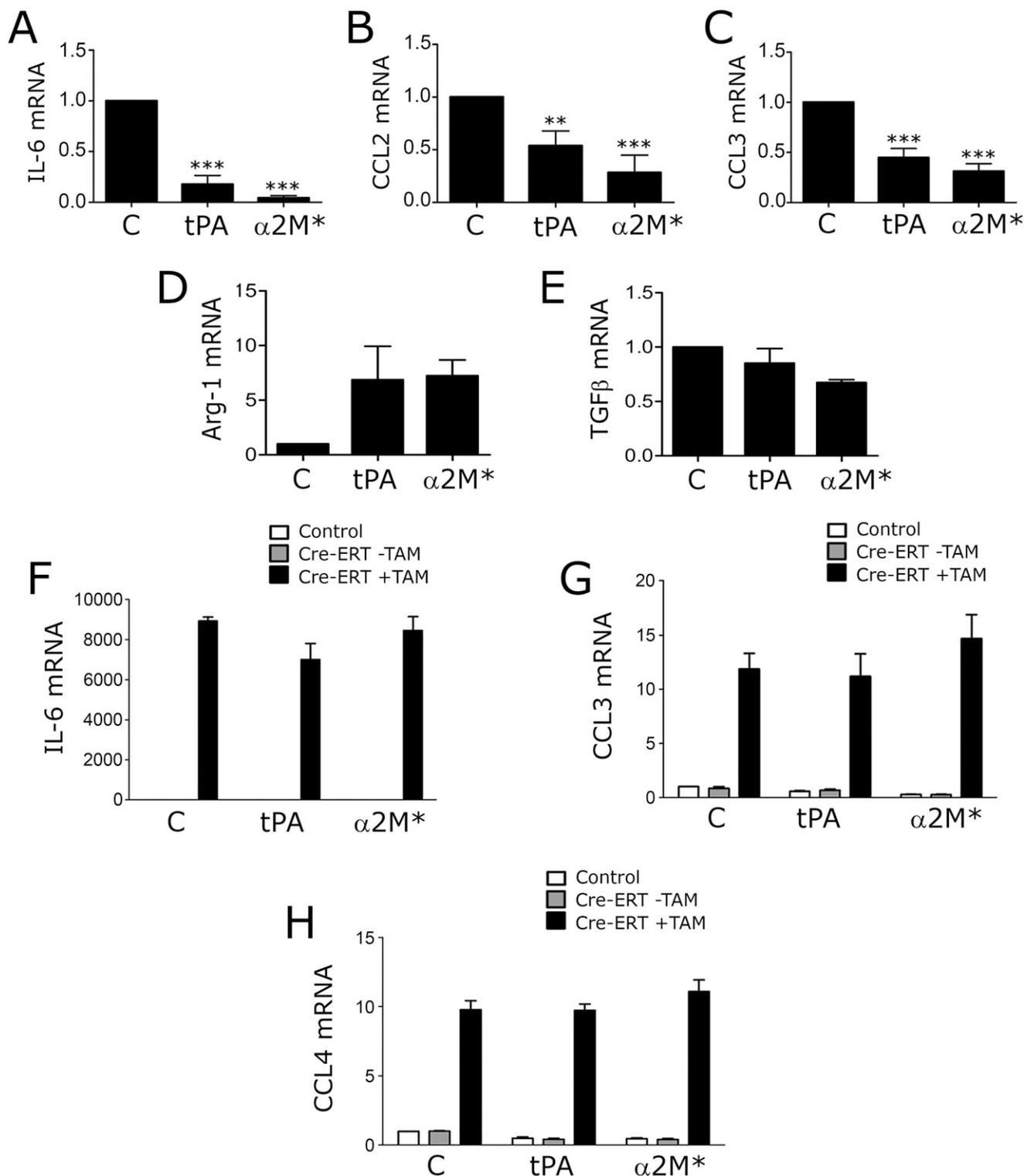


Fig. S3. $\alpha 2M^*$ and EI-tPA express antiinflammatory activity by an LRP1-dependent mechanism. (A–E) BMDMs were isolated from wild-type C57BL/6J mice and treated for 8 h with EI-tPA (12 nM), $\alpha 2M^*$ (10 nM), or vehicle (C). RTqPCR was performed to determine mRNA levels for IL-6, CCL2, CCL3, Arg-1, and TGF β (mean \pm SEM; $n = 4$; ** $P < 0.01$, *** $P < 0.001$; one-way ANOVA with Dunnett’s post hoc test). (F–H) LRP1 was deleted in BMDMs by treating cells isolated from Cre-ER^T-positive-LRP1^{fllox/fllox} mice with 5 μ M TAM (+TAM) for 7 d (solid bars). LRP1-expressing BMDMs included cells isolated from Cre-ER^T-negative-LRP1^{fllox/fllox} mice (open bars) and cells isolated from Cre-ER^T-positive-LRP1^{fllox/fllox} mice and treated with vehicle (–TAM, shaded bars). The cells were treated with EI-tPA, $\alpha 2M^*$, or vehicle (C) for 8 h. mRNA levels for IL-6, CCL3, and CCL4 were determined by RT-qPCR (mean \pm SEM; $n = 4$).

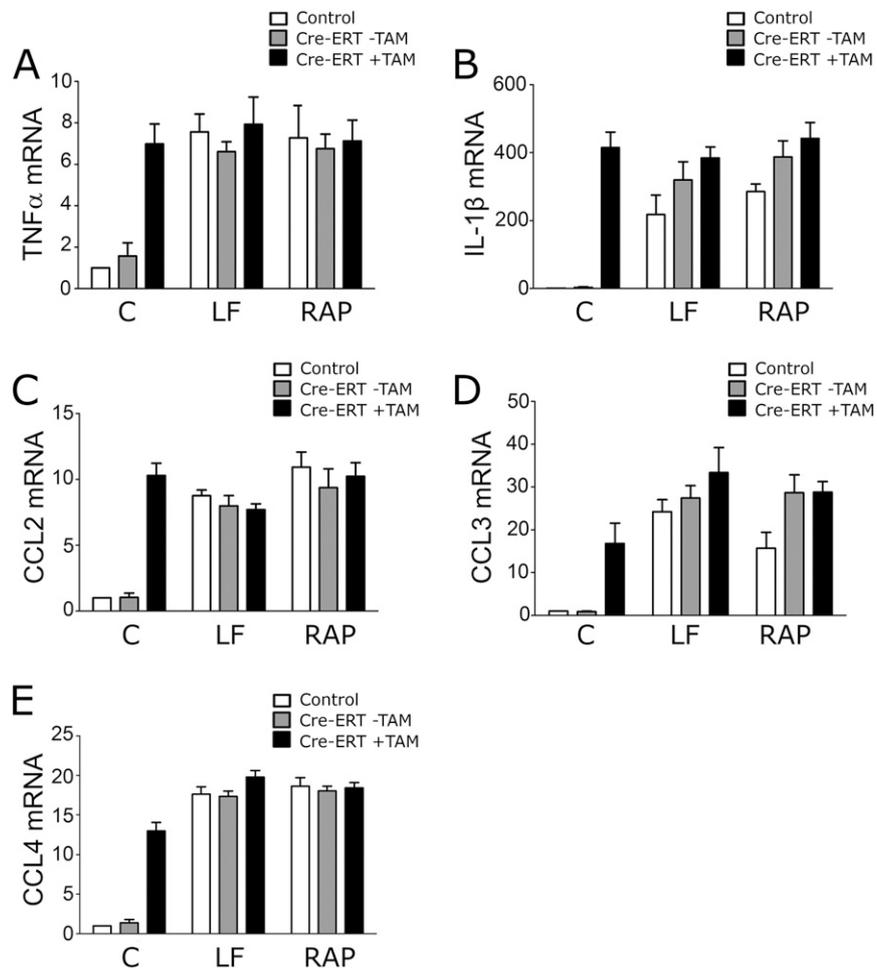


Fig. S4. RAP and LF do not further stimulate expression of proinflammatory mediators when *LRP1* is deleted. *LRP1* was deleted in BMDMs by treating cells isolated from Cre-ER^T-positive-*LRP1*^{fllox/fllox} mice with 5 μ M TAM (+TAM) for 7 d (solid bars). *LRP1*-expressing BMDMs included cells isolated from Cre-ER^T-negative-*LRP1*^{fllox/fllox} mice (open bars) and cells isolated from Cre-ER^T-positive-*LRP1*^{fllox/fllox} mice and treated with vehicle (-TAM, shaded bars). The cells were treated with LF (150 nM), RAP (150 nM), or vehicle (C). RT-qPCR was performed to determine expression of: (A) TNF α ; (B) IL-1 β ; (C) CCL2; (D) CCL3; and (E) CCL4 (mean \pm SEM; $n = 4$).

