Myeloid Cell Receptor LRP1/CD91 Regulates Monocyte Recruitment and Angiogenesis in Tumors

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Abstract

Recruitment of monocytes into sites of inflammation is essential in the immune response. In cancer, recruited monocytes promote invasion, metastasis, and possibly angiogenesis. LDL receptor-related protein (LRP1) is an endocytic and cell-signaling receptor that regulates cell migration. In this study, we isografted Pan02 pancreatic carcinoma cells into mice in which LRP1 was deleted in myeloid lineage cells. Recruitment of monocytes into orthotopic and subcutaneous tumors was significantly increased in these mice, compared with control mice. LRP1-deficient bone marrow–derived macrophages (BMDM) expressed higher levels of multiple chemokines, including, most prominently, macrophage inflammatory protein-1α/CCL3, which is known to amplify inflammation. Increased levels of CCL3 were detected in LRP1-deficient tumor-associated macrophages (TAM), isolated from Pan02 tumors, and in RAW 264.7 macrophage-like cells in which LRP1 was silenced. LRP1-deficient BMDMs migrated more rapidly than LRP1-expressing cells in vitro. The difference in migration was reversed by CCL3-neutralizing antibody, by CCR5-neutralizing antibody, and by inhibiting NF-κB with JSH-23. Inhibiting NF-κB reversed the increase in CCL3 expression associated with LRP1 gene silencing in RAW 264.7 cells. Tumors formed in mice with LRP1-deficient myeloid cells showed increased angiogenesis. Although VEGF mRNA expression was not increased in LRP1-deficient TAMs, at the single-cell level, the increase in TAM density in tumors with LRP1-deficient myeloid cells may have allowed these TAMs to contribute an increased amount of VEGF to the tumor microenvironment. Our results show that macrophage density in tumors is correlated with cancer angiogenesis in a novel model system. Myeloid cell LRP1 may be an important regulator of cancer progression. Cancer Res; 73(13); 3902-12. ©2013 AACR.

Introduction

Monocyte recruitment into sites of inflammation is controlled by locally produced chemokines (1). In addition, chemokines are involved in recruitment of monocytes into tumors, which may have a substantial effect on tumor progression (2, 3). When present in a cancer, macrophages show profound phenotypic variability. M1-like macrophages are cytolytic and express inflammatory mediators, including TNF-α and interleukin (IL)-12, reactive oxygen intermediates, and nitric oxide (3, 4). M2-like macrophages are involved in tissue remodeling and express different extracellular mediators, including TGF-β and platelet-derived growth factor (PDGF), which may support cancer cell survival, invasion, and metastasis (3–7). In many forms of cancer, tumor-associated macrophages (TAM) acquire M2-like properties owing to conditioning by factors released by the cancer cells (4). Because M2 TAMs express VEGF and proteases that function in tissue remodeling, these TAMs may support tumor angiogenesis (7). In clinical studies focused on diverse forms of human cancer, the abundance of TAMs correlates with disease progression (8–14). Understanding pathways that control monocyte recruitment in cancer is an important problem.

LDL receptor-related protein (LRP1) is a type 1 transmembrane receptor, which binds more than 40 ligands, mediating ligand endocytosis in clathrin-coated pits and cargo trafficking to lysosomes (15, 16). LRP1 also functions in phagocytosis of large particles and in the regulation of cell signaling (15, 17–19). The multifunctional nature of LRP1 is apparent when one considers its effects on cell migration. In fibroblasts, LRP1 inhibits cell migration by decreasing the cell-surface abundance of urokinase receptor (uPAR; refs. 20, 21). In macrophages, LRP1 may promote cell migration by facilitating Mac-1 trafficking when tissue-type plasminogen activator and its Serpin inhibitor, PAI-1, are available (22). In Schwann cells, LRP1 promotes cell migration by regulating the activation state of Rho-GTPases (23).

LRP1 gene deletion in mice is embryonically lethal (24). When LRP1 is deleted in myeloid cells, under the control of the LysM promoter, increased atherosclerosis is observed in different model systems (25, 26). Atherosclerotic lesions show an increase in macrophage density and increased levels of monocyte chemotactic protein (MCP-1)/CCL2 (25). Regulation of
CCL2 expression, in LRPI-deficient macrophages, reflects activation of the IKK–NF-κB signaling pathway (25, 27). Given the ubiquitous nature of this signaling pathway (28), we hypothesized that changes in macrophage signaling and physiology, observed in models of atherosclerosis, may also be observed in cancer.

In this study, our goal was to determine whether LRPI expression in monocytes and macrophages regulates their accumulation and activity in tumors in mice. We show that when LRPI is deleted in myeloid lineage cells, the density of TAMs in tumors formed by Pan02 pancreatic carcinoma cells is significantly increased. The increase in TAMs is accompanied by a significant increase in tumor angiogenesis. We have uncovered a novel mechanism that may underlie the ability of LRPI to regulate TAM accumulation in tumors, which is its ability to regulate macrophage inflammatory protein-1α/CCL3. This chemokine is known to amplify inflammation by increasing recruitment of monocytes into tissues that are already inflamed (1). Increased CCL3 expression, in LRPI-deficient bone marrow-derived macrophages (BMDM), promotes cell migration in vitro. Regulation of CCL3 by LRPI may represent an important pathway by which LRPI regulates TAM accumulation in cancers and cancer angiogenesis.

Materials and Methods

Antibodies and reagents

LRPI-specific antibodies that detect the α- and β-chain were obtained from Sigma-Aldrich. CCL3- and CCR5-specific antibodies were obtained from Abcam. Function-blocking antibody targeting CCL3 was procured from Thermo-Fisher Scientific. For immunofluorescence microscopy, Alexa 488-conjugated secondary antibody was obtained from Invitrogen. The NF-κB inhibitor, JSH-23, was from EMD Biosciences.

Mice

Mice in which the promoter and first 2 exons of the LRPI gene are flanked by loxP sites were generated by Rohmann and colleagues (29). We obtained mice that carry 2 copies of the floxed LRPI gene (LRPIflx/flx) and Cre recombinase under the control of the Lysozyme M (LysM) promoter, in the C57BL/6 background (25). For experiments, we bred LRPIflx/flx mice that are LysM-Cre-negative with LRPIflx/flx mice that are LysM-Cre-positive. Littermates in which myeloid cells were LRPI-expressing (mLRPI+ mice) or LRPI-deficient (mLRPI− mice) were studied. Littermates were born at a 50:50 ratio. All breeding protocols and experiments were approved by the University of California San Diego School of Medicine Institutional Animal Care and Use Committee.

Cell lines

RAW 264.7 cells were cultured in RPMI1640 supplemented with 10% FBS. RAW 264.7 cells, in which LRPI is constitutively silenced with short hairpin RNA (shRNA) have been previously described (30). Control cells were transfected with empty vector (pSUPER). Mouse Pan02 pancreatic adenocarcinoma cells were obtained from the National Cancer Institute Developmental Therapeutics Program Tumor Repository. Pan02 cells were periodically screened and found to be robustly immunopositive for mouse uPAR, immunonegative for human uPAR, and immunopositive for cytokertatin 19. Screening was last done when the studies reported here were concluded. Pan02 cells were cultured in low-glucose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and passaged no more than 5 times.

Isolation of BMDMs

Bone marrow cells were harvested from 6- to 10-week-old mLRPI+ and mLRPI− mice. The mice were euthanized. The femurs and tibiae were flushed with the long bones and cultured in 70% F12 medium with 10% FBS and 20% L929 cell-conditioned medium as a source of monocyte colony-stimulating factor. After 7 days of culturing, nonadherent cells were eliminated. Adherent cells, which were highly enriched in BMDMs, were recovered for studies. Final BMDM preparations were more than 97% F4/80-immunopositive, as determined by flow cytometry (n = 6).

Orthotopic and subcutaneous tumor isografts

Male mLRPI+ and mLRPI− mice (7 to 11 weeks old) were anesthetized with ketamine/xylazine and placed in the right lateral decubitus position. Flanks were wiped with Betadine and scrubbed with alcohol. The skin and peritoneum were incised longitudinally for 5 mm. The spleen was exteriorized and scrubbed with alcohol. The skin and peritoneum were exteriorized and scrubbed with alcohol. The spleen was exteriorized and scrubbed with alcohol. The spleen was returned to the peritoneum and the wound closed. Mice were euthanized 20 days later. Orthotopic tumors were harvested together with the intact pancreas.

Subcutaneous isografts were established by injecting 1 × 106 Pan02 cells in 100 μL of growth factor-reduced Matrigel (BD Biosciences) into the flank region. Tumors were harvested 30 days after inoculation.

Isolation of TAMs

Pan02 tumors were grown subcutaneously in mice (n = 6 per group), excised, and chopped into small pieces, which were treated with 800 U/mL Collagenase IV, 400 U DNase I, and 100 U/mL hyaluronidase in RPMI for 30 minutes. Cells were pelleted at 300 × g. After washing, cells were filtered through 100-μm and 45-μm cell strainers. CD11b+ cells were isolated using CD11b-microbeads (Miltenyi). For some experiments, recovered cells were allowed to adhere in Petri dishes to increase the fraction of TAMs.

Flow cytometry

CD11b+ cells from Pan02 tumors were incubated with anti-CD16 antibody (BD Biosciences) to block binding of other antibodies to endogenous Fc receptors. The cells were then treated with allophycocyanin-labeled F4/80-specific antibody or phycoerythrin-labeled CD206-specific antibody (eBioscience). Control cells were incubated with isotype-matched antibodies. Flow cytometry was carried out using a FACS-Canto II instrument. Results were analyzed using FlowJo.
software. The percentage of F4/80⁺ cells was multiplied by the number of CD11b⁺ cells in each tumor and then divided by the tumor weight to estimate the density of TAMs.

**Protein arrays**

Cytokines and chemokines were detected using the Mouse Cytokine Array Panel A Proteome Profiler (R&D Systems). BMDMs from mLRP¹⁺ and mLRP¹⁻ mice were allowed to condition SFM for 24 hours. Samples of conditioned medium were preincubated with detection solutions and then applied to membranes. Membranes were developed using enhanced chemiluminescence.

**Transwell migration assays**

Haptotactic cell migration assays were conducted using 24-well Transwell chambers. The underside of each membrane was coated with 10 µg/mL fibronectin (Millipore). BMDMs (5 × 10⁵) from mLRP⁺ and mLRP⁻ mice were added to the upper chamber. In some studies, cells were pretreated for 15 minutes with blocking antibodies targeting CCL3 (3 µg/mL), CCR5 (0.4 µg/mL) or with isotype-matched control antibodies. The same antibodies were added to the Transwell chambers. In other studies, BMDMs were pretreated with 20 µmol/L JSH-23 or with vehicle for 48 hours. Migration was conducted for 16 hours. Nonmigrating cells were removed from the upper surface using a cotton swab. The lower surface was stained with Hema 3 (Fisher Scientific). Stained membranes were mounted on microscope slides and imaged using a Leica DMIRE2 microscope. The number of migrated cells was determined on microscope slides and imaged using a Leica DMIRE2 microscope. At least 5 representative images of each section were selected by a blinded investigator and analyzed using ImageJ software. In control experiments, we determined that counting green-fluorescent macrophages and measuring the overall green fluorescence signal yielded equivalent results.

To quantitate angiogenesis, mean green fluorescence values were determined for at least 3 representative fields of each CD31-immunostained section using ImageJ software. As a negative control, we analyzed tissue sections that were incubated only with the secondary antibody.

**Quantitative RT-PCR**

Total RNA was extracted using the RNA EasyKit (Macherey-Nagel). cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Real-time quantitative RT-PCR (qRT-PCR) was carried out using TaqmanFast Universal PCR Mastermix 2x, TaqMan primers and probes for LRPI (30), CCL2 (Mm00441242_m1 CCL2), CCL3 (Mm00441258_m1 CCL3), CCL4 (Mm00443111_m1 CCL4), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm9999915_g1 GAPDH) as well as a StepOnePlus instrument from Applied Biosystems. Samples were probed for GAPDH mRNA as an internal normalization control. Experiments were conducted in triplicate with internal duplicate determinations.

**Immunoblot analysis**

Cell extracts were prepared in RIPA buffer containing Complete Protease Inhibitor Cocktail (Roche Diagnostics). Protein content was determined by bicinchoninic acid assay. Equal amounts of cellular protein were subjected to SDS-PAGE. Samples were electrotransferred to polyvinylidene difluoride (PVDF) membranes. Blocked membranes were incubated with LRPI- or CCL3-specific antibodies and then with horseradish peroxidase-conjugated secondary antibody. As a control for load, membranes were reprobed for tubulin. When conditioned medium samples were analyzed, we standardized the amount of medium subjected to SDS-PAGE by measuring the number of cells in each well using Hema 3 staining. Chromagen was eluted and quantitated by measuring Abs₆₅₀ nm. Absorbance was a linear function of cell number.

**In situ RNA analysis**

CCL3 mRNA was detected in tumor samples using RNA-scope, an RNA *in situ* hybridization method that permits signal amplification and background suppression (32). Tumor tissue sections (4.0 µm) were de-paraffinized and treated with protease according to the manufacturer’s instructions (Advanced Cell Diagnostics). CCL3-specific RNA target probe sets, provided by the manufacturer, targeted hps 23 through 771 of the CCL3 cDNA sequence. Following signal amplification, probe conjugated to horseradish peroxidase was imaged with 3,3′-diaminobenzidine (DAB). Sections were counterstained with DAPI-containing mounting solution.
hematoxylin. All steps of this procedure were carried out using a Ventana Discovery Ultra.

**Statistical analysis**

Statistical analysis was conducted using Microsoft Excel and GraphPad Prism. In cell-migration experiments, we used a Student unpaired t test, 1-way ANOVA test, or 2-way ANOVA test and Tukey post hoc test. TAM content and the extent of angiogenesis were analyzed using a Student unpaired t test. P values less than 0.05 were considered statistically significant.

**Results**

**LRP1 deficiency in myeloid cells promotes monocyte recruitment into tumors in vivo**

Monocyte recruitment into tumors is an important determinant of tumor progression (4–14). LRP1 is described as a regulator of cell migration (20–23) and inflammation (25, 27, 30, 33). To test whether LRP1 expression in monocytes and macrophages regulates recruitment of monocytes into tumors, we studied mice in which LRP1 is deleted in myeloid lineage cells (mLRP1− mice). To show that LRP1 was deleted in macrophages from mLRP1− mice, we isolated BMDMs and conducted immunoblotting experiments. The 515-kDa LRP1 α-chain and the 85-kDa LRP1 β-chain were readily detected in BMDMs from control mLRP1+ mice and essentially absent in BMDMs from mLRP1− mice (Fig. 1A).

Next, we inoculated mouse PanO2 pancreatic adenocarcinoma cells directly into the pancreas in mLRP1+ and mLRP1− syngeneic mice (n = 4/group). Necropsies were carried out 20 days later, at which time the mean tumor mass was not significantly different in the 2 cohorts. F4/80+ macrophages were detected by immunofluorescence microscopy and

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**Figure 1.** Recruitment of LRP1-deficient monocytes to PanO2 tumors is increased. A, extracts of BMDMs from 3 separate mLRP1+ and mLRP1− mice were subjected to immunoblot analysis to detect LRP1 α-chain, β-chain, and tubulin. LRP1 α-chain levels were determined by densitometry (*, *P < 0.01). B, macrophage infiltration of orthotopic PanO2 tumors in mLRP1+ and mLRP1− mice was determined by F4/80 immunofluorescence microscopy. TAMs were quantitated. Results obtained by analyzing tumors in different mice are summarized (**, **P < 0.05). C, macrophage infiltration of subcutaneous PanO2 tumors in mLRP1+ and mLRP1− mice was determined as in B. D, flow cytometry was carried out to determine F4/80+, CD11b− cells in mLRP1+ or mLRP1− mice. E, the percentage of F4/80+ cells that were CD206+ is shown for mLRP1+ and mLRP1− mice.
Figure 1B shows that the number of TAMs was significantly increased in tumors recovered from mLRP1- mice ($P < 0.05$). Subcutaneous PanO2 tumors also were established in mLRP1+ and mLRP1- mice and allowed to grow for 30 days. Figure 1C shows that the number of F4/80-positive TAMs was significantly increased in subcutaneous tumors in mLRP1- mice ($n = 5–6$/cohort; $P < 0.05$).

As a second experimental approach, subcutaneous tumors ($n = 6$/cohort) were digested to isolate cells. CD11b+ cells were recovered using magnetic beads and subjected to flow cytometry to detect F4/80+ cells. Figure 1D shows that the number of F4/80+ macrophages was significantly increased in tumors from mLRP1- mice, compared with mLRP1+ mice. The same cells were subjected to flow cytometry to detect the M2 macrophage marker, CD206. A significant change in the percentage of CD11b-F4/80+ cells that were also CD206-immunopositive was not observed (Fig. 1E).

LRP1 regulates chemokine expression by BMDMs

To screen for novel mechanisms that may be responsible for the increase in recruitment of LRP1-deficient monocytes into tumors, we isolated BMDMs from mLRP1+ and mLRP1- mice. The BMDMs were allowed to condition SFM, which was screened for expression of 40 cytokines, chemokines, and other extracellular mediators. To model different extents of macrophage activation that may be observed in vivo, the BMDMs were treated with 100 ng/mL lipopolysaccharide (LPS) for 24 hours or with vehicle.

CCL2 was substantially increased in medium conditioned by LRP1-deficient BMDMs, in the absence of LPS, compared with LRP1-expressing cells (Fig. 2A), as previously reported (25, 27). LPS substantially increased CCL2 secretion by LRP1-expressing BMDMs, eliminating the difference in CCL2 produced by LRP1-expressing and -deficient cells (Fig. 2B). In addition, CCL3 and MIP-1B/CCL4 were increased in medium conditioned by LRP1-deficient BMDMs. This result was observed with or without LPS treatment. Because the absolute level of CCL3 in conditioned SFM was higher than that of CCL4, we conducted independent immunoblotting experiments to validate the CCL3 results. BMDMs from mLRP1+ and mLRP1- mice were treated with LPS or vehicle for 24 hours. Conditioned medium was isolated for analysis. Figure 2C shows that, under all the experimental conditions, LRP1-deficient BMDMs produced substantially more CCL3 than LRP1-expressing cells.

CCR5 is a G-protein-coupled receptor for CCL3 (1). As shown in Fig. 2D, BMDMs from mLRP1- mice had higher levels of CCR5 compared with LRP1-expressing BMDMs. As a control, we showed that CCR1 was unchanged in LRP1-deficient BMDMs. LRP1 is known to regulate levels of diverse proteins in the plasma membrane proteome (33).
LRP1 regulates CCL3 expression in RAW 264.7 cells

To confirm our results in a second model system, we undertook experiments with RAW 264.7 macrophage-like cells. RAW 264.7 cells in which LRP1 gene expression is stably silenced with shRNA have been previously described (30). Figure 3A shows an immunoblot analysis comparing the level of 85-kDa β-chain in silenced cells and in control cells transfected with empty vector (pSUPER). Expression of CCL2, CCL3, and CCL4 was compared in LRP1 gene-silenced and control RAW 264.7 cells. A significant increase ($P < 0.05$) in CCL3 mRNA was observed in the LRP1 gene-silenced cells (Fig. 3B). Because we previously showed that IKK–NF-κB signaling may be activated in LRP1-deficient macrophages (27), we examined the ability of JSH-23, which inhibits nuclear translocation of NF-κB, to reverse the effects of LRP1 deficiency on CCL3 mRNA expression. When LRP1 gene-silenced RAW 264.7 cells were treated with 20 μmol/L JSH-23, CCL3 expression was decreased to the level detected in LRP1-expressing cells.

Next, we examined CCL3 accumulation in SFM conditioned by LRP1 gene-silenced and control RAW 264.7 cells. Figure 3C shows that CCL3 accumulated at higher levels in SFM conditioned by LRP1 gene-silenced cells. When the cells were cultured in the presence of 20 μmol/L JSH-23, the level of CCL3 in SFM was substantially reduced. Three separate immunoblot analyses were subjected to densitometry analysis. The results are summarized.

CCL3 promotes migration of LRP1-deficient BMDMs

To test whether CCL3 may be responsible for the increase in monocyte recruitment into tumors in mLRP1−/− mice, first we examined BMDM cell migration in vitro using a Transwell model system. Transwell membranes were coated on the underside surface with fibronectin. In 16 hours, 1.2 ± 0.3% of the BMDMs isolated from mLRP1+/− mice migrated to the underside surfaces of the membranes (mean ± SEM, n = 4). Figure 4A shows that migration of BMDMs, isolated from mLRP1−/− mice, was significantly increased ($P < 0.05$).

Next, neutralizing antibodies that target CCL3 or CCR5 were added to Transwells. Isotype-matched antibodies, which were added in control studies, had no effect on cell migration compared with that observed when no antibody was added (shown in Fig. 4A). Figure 4B shows that CCL3- and CCR5-specific antibodies did not affect migration of LRP1-expressing BMDMs. By contrast, both neutralizing antibodies significantly decreased migration of LRP1-deficient BMDMs, so that these cells migrated in a similar manner to that of LRP1-expressing cells.

Our results suggested a model in which an extracellular mediator (CCL3) produced by LRP1-deficient BMDMs...
functions in an autocrine pathway to promote cell migration. To further test this model, we studied random BMDM cell migration using time-lapse microscopic imaging. Figure 4C shows cell-migration maps for LRP1-expressing and -deficient BMDMs, which were allowed to condition medium for 24 hours before initiating imaging. As shown in Fig. 4D, migration of LRP1-deficient BMDMs was significantly increased, compared with LRP1-expressing cells (P < 0.005). When time-lapse imaging was initiated immediately to ensure the cells did not have time to precondition the medium, LRP1-deficient BMDMs failed to show a significant increase in migration (Fig. 4E).

Because our results suggested that CCL3 may be produced downstream of NF-κB in LRP1-deficient cells, we tested whether JSH-23 regulates BMDM cell migration. Figure 4F shows that LRP1-expressing BMDMs that were treated with JSH-23 migrated at about the same rate as cells that were treated with vehicle. By contrast, JSH-23 significantly attenuated migration of LRP1-deficient BMDMs (P < 0.05), so that these cells migrated at a rate that was equivalent to that shown by LRP1-expressing cells.

**LRP1-deficient TAMs express CCL3**

To test whether CCL3 is produced in tumors formed by Pan02 cells, we applied RNA in situ hybridization (32). Figure 5A shows that robust CCL3 mRNA expression was restricted to a subset of the cells in the tumors, which, at high magnification,
studies to test whether angiogenesis is increased in these extent than LRP1-expressing macrophages, we conducted LRP1-de accompanied by an increase in angiogenesis (4). mLRP1 detected in medium conditioned by cells isolated from mLRP1 (100 ng/mL), cells isolated from mLRP1 +/− mice showed plumper with increased DAB oxidation, compared with the cells present in tumors formed in mLRP1 +/− mice.

Next, we isolated TAM-enriched cell preparations from tumors formed in mLRP1 +/− and mLRP1 −/− mice, using CD11b-microbeads. Cells isolated from mLRP1 −/− mice showed a 60% decrease in LRP1 mRNA, compared with cells from mLRP1 +/− mice. When tumor-derived CD11b + cells were cultured ex vivo for 24 hours, in the presence or absence of LPS (100 ng/mL), cells isolated from mLRP1 −/− mice showed more than a 2-fold increase in CCL3 mRNA (Fig. 5B). CCL3 also was detected in medium conditioned by cells isolated from mLRP1 +/− mice following LPS treatment (Fig. 5C). Under equivalent conditions, we did not detect CCL3 in medium conditioned by cells from mLRP1 +/− mice. These results show that the effects of LRP1 on expression of CCL3 in BMDMs and RAW 264.7 cells are conserved in TAMs.

Tumors in mLRP1 −/− mice show increased angiogenesis

Increased infiltration of tumors by macrophages may be accompanied by an increase in angiogenesis (4–7). Because LRP1-deficient macrophages accumulate in tumors to a greater extent than LRP1-expressing macrophages, we conducted studies to test whether angiogenesis is increased in these tumors. Orthotropic PanO2 tumors isolated from mLRP1 −/− and mLRP1 +/− mice were immunostained for the endothelial cell marker, CD31. Figure 6A shows that the density of CD31 + blood vessels was significantly increased (P < 0.05) in tumors isolated from mLRP1 −/− mice. Equivalent results were obtained when we analyzed subcutaneous PanO2 isografts (Fig. 6B).

To test whether the increase in angiogenesis reflected increased expression of VEGF, we isolated CD11b + cells from subcutaneous tumors. Figure 6C shows that VEGF expression by LRP1-deficient CD11b + cells was not significantly increased compared with LRP1-expressing cells. However, when VEGF mRNA expression was corrected for the increased density of TAMs in tumors formed in mLRP1 −/− mice, the results suggest that these TAMs may contribute an increased amount of VEGF to the tumor microenvironment (Fig. 6D).

Discussion

In this study, we showed that LRP1-deficient monocytes are recruited in increased numbers into PanO2 tumors. LRP1 has been described as a regulator of cell migration, for example, by controlling the cell-surface abundance of uPAR (20, 21); however, this type of mechanism does not allow for paracrine regulation of cell migration. We focused on the ability of LRP1 to regulate the release of soluble mediators, which may function as chemoattractants to circulating monocytes. A mechanism of this nature offers the opportunity for amplification of inflammation. Macrophages that have entered an inflamed tissue may participate in recruitment of additional monocytes.

One factor, which is expressed by macrophages at increased levels when LRP1 is deficient, is CCL2 (25, 27). Expression of CCL2 by cancer cells promotes monocyte migration and recruitment into malignancies (34, 35). In addition, CCL2 may promote cancer cell migration (36–38). The original studies showing that LRP1 deficiency in macrophages is associated with increased CCL2 expression focused on atherosclerosis (25, 27). We confirmed that CCL2 expression is increased in BMDMs isolated from mLRP1 −/− mice. After treatment with LPS, increased expression of CCL2 by LRP1-deficient BMDMs was no longer observed. This result is most likely explained by the fact that LPS promotes LRP1 shedding from LRP1-expressing cells (30). LRP1 thus shed is biologically active, inducing expression of CCL2 in macrophage-like cells (30). In experiments that are not shown, CCL2 function-blocking antibody did not regulate migration of LRP1-deficient BMDMs in vitro. Nevertheless, it cannot be ruled out that increased expression of CCL2 by LRP1-deficient macrophages contributes to the increase in TAM density observed in PanO2 tumors in mLRP1 −/− mice.

We report for the first time that expression of CCL3 and CCL4 is increased in BMDMs when LRP1 is deficient. These chemokines are involved in transendothelial cell migration and in activation of diverse inflammatory cells, including monocytes (1, 39–41). CCL3 plays an important role in amplifying inflammation. Our results indicate that attenuation of CCL3 expression constitutes a newly identified pathway by which LRP1 regulates cell migration in vitro. We propose that LRP1 regulates the CCL3-CCL5 system to limit accumulation of TAMs in cancer. When LRP1 is deficient in monocytes in vivo,
increased expression of CCL3 by macrophages that initially enter a tumor may lead to increased recruitment of additional monocytes. By this mechanism, local inflammation may be amplified.

We confirmed that LRP1 deficiency is associated with increased CCL3 expression in a second model system: RAW 264.7 cells. In these cells, increased CCL3 expression was driven downstream of NF-kB, as was previously described for CCL2 (27). In BMDMs, targeting NF-kB neutralized the migration advantage associated with LRP1 deficiency, presumably by inhibiting CCL3 secretion. Different models have been proposed regarding the mechanism by which LRP1 regulates NF-kB signaling (27, 33); however, it is clear that when this pathway is activated, the potential exists for increased expression of multiple inflammatory mediators (28). Our evidence suggests that regulation of expression of inflammatory mediators by LRP1 may be important in cancer.

Using mLRP1−/− mice as a model system, we confirmed an important paradigm, which is the association of TAM density in tumors with tumor angiogenesis (4–7). Tumors formed in mLRP1−/− mice showed increased blood vessel density. A significant increase in VEGF mRNA expression was not observed in TAMs isolated from mLRP1−/− mice; however, the increase in TAM density in tumors formed in mLRP1−/− mice may have allowed these macrophages to collectively contribute an increased amount of VEGF to the tumor microenvironment. Pan02 cancer cells do not metastasize under normal conditions, and we saw no evidence of metastasis to the lungs in mLRP1−/− mice. However, the increase in TAM density and angiogenesis observed in tumors formed in mLRP1−/− mice suggest that macrophage LRP1 may be linked to cancer metastasis in some forms of cancer. Control of tumor angiogenesis by TAMs is an important example of how the tumor microenvironment regulates cancer progression.

The major goal of this study was to understand how LRP1 regulates macrophage physiology in cancer. We have shown that LRP1 suppresses a chemokine system that may otherwise promote cancer progression. In addition, we have linked TAM density to tumor angiogenesis in a novel model system. The relevance of our work to human malignancy may be found in multiple studies that describe LRP1 as a highly regulated gene product in monocytes and macrophages (30, 42–47). Changes in the cell-surface abundance of LRP1 result from regulation of transcription, shedding, and degradation. Combinations of extracellular mediators may be sufficient to substantially downregulate LRP1 in TAMs (30, 43, 44). We now understand that a consequence of LRP1 downregulation may be the activation of the CCL3 system and amplification of inflammation. Preventing LRP1 downregulation in myeloid...
cells may limit monocyte recruitment to tumors and cancer angiogenesis.

Disclosure of Potential Conflicts of Interest
No conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: N.D. Staudt, M. Jo, J. Hu, J.M. Bristow, A. Gaultier, S.L. Gonias
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