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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 PanO2 pancreatic carcinoma cells into mice in which LRP1 is 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 (BMDMs) expressed higher levels of multiple chemokines, including, most prominently, macrophage inflammatory protein-1 α /CCL3, which is known to amplify inflammation. Increased levels of CCL3 were detected in LRP1-deficient tumor-associated macrophages (TAMs), isolated from PanO2 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 κ B with JSH-23. Inhibiting NF κ B 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 demonstrated 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 demonstrate 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.

Introduction

Monocyte recruitment into sites of inflammation is controlled by locally produced chemokines (1). Chemokines also are involved in recruitment of monocytes into tumors, which may have a substantial effect on tumor progression (2, 3). Once present in a cancer, macrophages demonstrate profound phenotypic variability. M1-like macrophages are cytolytic and express inflammatory mediators, including TNF α and IL-12, reactive oxygen

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intermediates, and nitric oxide (3, 4). M2-like macrophages are involved in tissue remodeling and express different extracellular mediators, including TGF β and PDGF, which may support cancer cell survival, invasion, and metastasis (3-7). In many forms of cancer, tumor-associated macrophages (TAMs) acquire M2-like properties due 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 over 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 considering its effects on cell migration. In fibroblasts, LRP1 inhibits cell migration by decreasing the cell-surface abundance of uPAR (20, 21). In macrophages, LRP1 may promote cell migration by facilitating the 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 embryonic 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 demonstrate an increase in macrophage density and increased levels of monocyte chemotactic protein (MCP-1)/CCL2 (25). Regulation of CCL2 expression, in LRP1-deficient macrophages, reflects activation of the IKK-NF κ B 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, also may be observed in cancer.

In this study, our goal was to determine whether LRP1 expression in monocytes and macrophages regulates their accumulation and activity in tumors in mice. We show that when LRP1 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 LRP1 to regulate TAM accumulation in tumors, which is its ability to regulate macrophage inflammatory protein-1 α /CCL3. This chemokine is known to amplify inflammation by increasing recruitment of monocytes into tissues that are already inflamed (1). Increased CCL3 expression, in LRP1-deficient bone marrow-derived macrophages (BMDMs), promotes cell migration *in vitro*. Regulation of CCL3 by LRP1 may represent an important pathway by which LRP1 regulates TAM accumulation in cancers and cancer angiogenesis.

Materials and Methods

Antibodies and reagents

LRP1-specific antibodies that detect the α -chain and β -chain were from Sigma-Aldrich. CCL3-specific antibody and CCR5-specific antibody were from Abcam. Function-blocking antibody targeting CCL3 was from Thermo-Fisher Scientific. For immunofluorescence (IF) microscopy, F4/80-specific antibody (CI:A3-1) was from Abcam and CD31-specific antibody was from Bio-Legend. Alexa 488-conjugated secondary antibody was from Invitrogen. The NF κ B inhibitor, JSH-23, was from EMD Biosciences.

Mice

Mice in which the promoter and first two exons of the LRP1 gene are flanked by loxP sites were generated by Rohlmann et al (29). We obtained mice that carry two copies of the floxed LRP1 gene (LRP1^{flox/flox}) and *Cre* recombinase under the control of the Lysozyme M (LysM) promoter, in the C57BL/6 background (25). For experiments, we bred LRP1^{flox/flox} mice that are LysM-*Cre*-negative with LRP1^{flox/flox} mice that are LysM-*Cre*-positive. Littermates in which myeloid cells were LRP1-expressing (mLRP1+ mice) or LRP1-deficient (mLRP1- mice) were studied. Littermates were born at a 50:50 ratio. All breeding protocols and experiments were approved by the UCSD Institutional Animal Care and Use Committee.

Cell lines

RAW 264.7 cells were cultured in RPMI 1640 supplemented with 10% FBS. RAW 264.7 cells, in which LRP1 is constitutively silenced with shRNA are previously described (30). Control cells were transfected with empty vector (pSUPER). Mouse PanO2 pancreatic adenocarcinoma cells were obtained from the National Cancer Institute Developmental Therapeutics Program Tumor Repository. PanO2 cells were periodically screened and found to be robustly immunopositive for mouse urokinase receptor, immunonegative for human urokinase receptor, and immuno-positive for cytokeratin 19. Screening was last performed when the studies reported here were concluded. PanO2 cells were cultured in low-glucose DMEM supplemented with 10% FBS and passaged no more than five times.

Isolation of BMDMs

Bone marrow cells were harvested from 6-10 week old mLRP1+ and mLRP1- mice. The mice were euthanized. The femurs and tibiae were recovered from both rear legs. Bone marrow cells were flushed from 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, non-adherent cells were eliminated. Adherent cells, which were highly enriched in BMDMs, were recovered for studies. Final BMDM preparations were >97% F4/80 immunopositive, as determined by flow cytometry (n=6).

Orthotopic and subcutaneous tumor isografts

Male mLRP1+ and mLRP1- mice (7-11 week old) were anesthetized with ketamine/xylozine and placed in the right lateral decubitus position. Flanks were swiped with Betadine and scrubbed with alcohol. The skin and peritoneum were incised longitudinally for 5 mm. The spleen was exteriorized allowing access to the pancreas. PanO2 cells (5×10^5) in 20 μ L of serum-free medium (SFM) were injected into the pancreatic head. 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 1×10^6 PanO2 cells in 100 μ L of growth factor-reduced Matrigel (BD Bioscience) into the flank region. Tumors were harvested 30 days after inoculation.

Isolation of TAMs

PanO2 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 min. Cells were pelleted at 300 x *g*. After washing, cells were filtered through 100 μ m and 45 μ m 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 PanO2 tumors were incubated with anti-CD16 antibody (BD Bioscience) 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 performed 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 h. Samples of conditioned medium were pre-incubated with detection solutions and then applied to membranes. Membranes were developed using enhanced chemiluminescence.

Transwell migration assays

Haptotactic cell migration assays were performed using 24-well Transwell chambers. The underside of each membrane was coated with 10 µg/ml fibronectin (Millipore). BMDMs (5×10^5) from mLRP⁺ and mLRP⁻ mice were added to the upper chamber. In some studies, cells were pre-treated for 15 min 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 pre-treated with 20 µM JSH-23 or with vehicle for 48 h. Migration was conducted for 16 h. Non-migrating 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 in four representative fields, chosen by a blinded investigator, using ImageJ software. Three separate membranes were analyzed for each condition. Experiments were performed at least in triplicate.

Random cell migration assay

Tissue culture wells (35 mm) were coated with 10 µg/mL fibronectin. BMDMs (1.5×10^6) were seeded in each well. The following day, cells were washed with SFM and re-equilibrated in 2 mL of serum-free DMEM containing 25 µM HEPES, pH 7.4. A thin layer of mineral oil was layered over the medium to limit evaporation. In some experiments, imaging was initiated immediately. In other studies, the cells were allowed to condition SFM for 24 h before initiating imaging. Phase contrast images were collected using a 10x objective every 10 min for 12 h. During the imaging period, the cells were maintained at 37°C. Images were collected using a Nikon Eclipse Ti microscope with Nikon Elements acquisition software. Time-lapse images were analyzed using Metamorph software as previously described (31).

IF microscopy

Orthotopic and subcutaneous PanO2 cell tumors were excised, weighed, and cryofrozen in Tissuetek (Sakura Finetek). Tumors were sectioned at 5 µm for F4/80 immunostaining or at 10 µm for CD31 immunostaining. Tumor tissue sections were fixed in 4% formalin and then treated with 0.2% Triton X-100. Non-specific antibody binding was blocked with 5% goat serum and anti-CD16/Fc receptor antibody (1:1000). Sections were then incubated with anti-F4/80 antibody (1:2000) or anti-CD31 antibody (1:250) followed by Alexa 488-conjugated

secondary antibody (1:2000). Sections were mounted in DAPI-containing mounting solution. Microscopy was performed using a Leica DMIRE2 microscope. At least five 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.

qPCR

Total RNA was extracted using the RNA EasyKit (Macherey-Nagel, Bethlehem, PA). cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Real-time qPCR was performed using TaqmanFast Universal PCR Mastermix 2x, Taqman primers and probes for LRP1 (30), CCL2 (Mm00441242_m1 CCL2), CCL3 (Mm00441258_m1 CCL3), CCL4 (Mm00443111_m1 CCL4) and GAPDH (Mm99999915_g1 GAPDH) and a StepOnePlus instrument from Applied Biosystems. Samples were probed for GAPDH mRNA as an internal normalization control. Experiments were performed 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 PVDF membranes. Blocked membranes were incubated with LRP1-specific or CCL3-specific antibody and then with HRP-conjugated secondary antibody. As a control for load, membranes were re-probed 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 Abs_{650 nm}. Absorbance was a linear function of cell number.

In situ RNA analysis

CCL3 mRNA was detected in tumor samples using RNAscope, an RNA *in situ* hybridization method that permits signal amplification and background suppression (32). Tumor tissue sections (4.0 μ m) were de-paraffinized, and treated with protease, as described by the manufacturer (Advanced Cell Diagnostics). CCL3-specific RNA target probe sets, provided by the manufacturer, targeted bps 23-771 of the CCL3 cDNA sequence. Following signal amplification, probe conjugated to horseradish peroxidase was imaged with 3,3'-diaminobenzidine (DAB). Sections were counterstained with hematoxylin. All steps of this procedure were performed using a Ventana Discovery Ultra.

Statistical analysis

Statistical analysis was performed using Microsoft Excel and GraphPad Prism. In cell migration experiments, we used a Student's unpaired t-test, one-way-ANOVA test, or two-way-ANOVA test and Tukey's post-hoc test. TAM content and the extent of angiogenesis were analyzed using a Students 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 demonstrate that LRP1 was deleted in macrophages from mLRP1⁻ mice, we isolated BMDMs and performed 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 performed 20 days later, at which time the mean tumor mass was not significantly different in the two cohorts. F4/80-positive macrophages were detected by IF microscopy and counted. Fig. 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. Fig. 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-positive cells. Fig. 1D shows that the number of F4/80-positive 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-positive 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 h 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-positive BMDMs, eliminating the difference in CCL2 produced by LRP1-expressing and -deficient cells (Fig. 2B). CCL3 and MIP-1 β /CCL4 also 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 CCL4, we performed independent immunoblotting experiments to validate the CCL3 results. BMDMs from mLRP1⁺ and mLRP1⁻ mice were treated with LPS or vehicle for 24 h. Conditioned medium was isolated for analysis. Fig. 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 demonstrated 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 are previously described (30). Fig. 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 demonstrated that IKK-NF κ B signaling may be activated in LRP1-deficient macrophages (27), we examined the ability JSH-23, which inhibits nuclear translocation of NF κ B, to reverse the effects of LRP1 deficiency on CCL3 mRNA expression. When LRP1 gene-silenced RAW 264.7 cells were treated with 20 μ M 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. Fig. 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 μ M 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 h, $1.2 \pm 0.3\%$ of the BMDMs isolated from mLRP1⁺ mice migrated to the underside surfaces of the membranes (mean $\pm$ SEM, $n = 4$). Fig. 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). Fig. 4B shows that CCL3-specific and CCR5-specific antibody 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 similarly to 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 microscopy imaging. Fig. 4C shows cell migration maps for LRP1-positive and -deficient BMDMs, which were allowed to condition medium for 24 h prior to 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, so the cells did not have time to pre-condition the medium, LRP1-deficient BMDMs failed to demonstrate a significant increase in migration (Fig. 4E).

Because our results suggested that CCL3 may be produced downstream of NF κ B in LRP1-deficient cells, we tested whether JSH-23 regulates BMDM cell migration. Fig. 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 demonstrated by LRP1-expressing cells.

LRP1-deficient TAMs express CCL3

To test whether CCL3 is produced in tumors formed by PanO2 cells, we applied RNA *in situ* hybridization (32). Fig. 5A shows that robust CCL3 mRNA expression was restricted to a subset of the cells in the tumors, which, at high magnification, demonstrated macrophage-like morphology. Our method used DAB development to mark CCL3 mRNA. Many of the CCL3 mRNA-positive cells identified in tumors formed in mLRP1⁻ mice appeared 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 demonstrated a 60% decrease in LRP1 mRNA, compared with cells from mLRP1⁺ mice. When tumor-derived CD11b⁺ cells were cultured *ex vivo* for 24 h, in the presence or absence of LPS (100 ng/ml), cells isolated from mLRP1⁻ mice demonstrated 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 demonstrate that the effects of LRP1 on expression of CCL3 in BMDMs and RAW 264.7 cells are conserved in TAMs.

Tumors in mLRP1⁻ mice demonstrate 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 performed studies to test whether angiogenesis is increased in these tumors. Orthotopic PanO2 tumors isolated from mLRP1⁻ and mLRP1⁺ mice were immunostained for the endothelial cell marker, CD31. Fig. 6A shows that the density of CD31-positive 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. Fig. 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 demonstrated 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). CCL2 also may promote cancer cell migration (36-38). The original studies demonstrating 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). Shed LRP1 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-CCR5 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 κ B, as was previously described for CCL2 (27). In BMDMs, targeting NF κ B 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 κ B signaling (27, 33); however, it is clear that once 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 LRP1⁻ 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. PanO2 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. We have also 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 down-regulate LRP1 in TAMs (30, 43, 44). We now understand that a consequence of LRP1 down-regulation may be activation of the CCL3 system and amplification of inflammation. Preventing LRP1 down-regulation in myeloid cells may limit monocyte recruitment to tumors and cancer angiogenesis.

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Figure 1.

Recruitment of LRP1-deficient monocytes to PanO2 tumors is increased. A, Extracts of BMDMs from three 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 IF 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 panel B. D, Flow cytometry was performed to

determine F4/80-positive, CD11b+ cells in mLRP1+ or mLRP1– mice. E, The percentage of F4/80-positive cells that were CD206-positive is shown for mLRP1+ and mLRP1– mice.

Figure 2.

Chemokine expression is increased in LRP1-deficient BMDMs. A, BMDMs from mLRP1+ and mLRP1- mice were allowed to condition SFM. Expression of extracellular mediators was determined using an Array system. B, BMDMs were treated with LPS. Conditioned medium was analyzed using the Array system. Mediators detected in SFM and possibly regulated by LRP1 but not discussed further include CXCL10, TIMP-1, and CCL-12 (ND, not detected). C, BMDMs were treated with LPS or vehicle for 24 h. Conditioned medium was recovered for immunoblot analysis. D, Cell extracts were prepared from BMDMs isolated from mLRP1+ and mLRP1- mice and subjected to immunoblot analysis to detect CCR5 and tubulin.

Figure 3.

LRP1 regulates CCL3 expression in RAW 264.7 cells by an NF κ B-dependent pathway. A, Expression of LRP1 by RAW 264.7 cells in which LRP1 is silenced and by control cells (pSUPER). B, RAW 264.7 cells in which LRP1 is expressed (+) or silenced (-) were treated with JSH-23 or vehicle for 48 h. Expression of the indicated mRNAs was determined by qPCR (*, $p < 0.05$; NS, not significant). C, LRP1-expressing (+) or gene-silenced (-) RAW 264.7 cells conditioned SFM for 24 h. The cells were treated with JSH-23, vehicle (DMSO), or un-treated. Immunoblot analysis was performed to detect CCL3. The results of three experiments were analyzed by densitometry and summarized (*, $p < 0.05$).

Figure 4.

LRP1-deficient BMDMs demonstrate increased cell migration due to activation of the CCL3-CCR5 system. A, Migration of LRP1-expressing and -deficient BMDMs was compared in Transwell experiments (*, $p < 0.05$). B, Migration of BMDMs was studied in the presence of CCL3-neutralizing antibody, CCR5-neutralizing antibody, or isotype-matched control antibody. Each data point shows results obtained with BMDMs from a separate mLRP1+ or mLRP1- mouse. C, Random cell migration maps for LRP1-expressing and LRP1-deficient BMDMs, which pre-conditioned SFM for 24 h before initiating imaging. D, Results presented in panel C were summarized ($n = 20$, ***, $p < 0.005$). E, Random cell migration was imaged without allowing pre-conditioning of the medium (ns, not statistically

significant). F, BMDMs from mLRP1+ and mLRP1– mice were treated with 20 μ M JSH-23 or vehicle and studied in Transwell migration experiments. JSH-23 significantly decreased migration of BMDMs isolated from mLRP1– mice ($p<0.05$).

Figure 5.

LRP1-deficient TAMs express CCL3. A, CCL3 mRNA was detected in tumors by *in situ* hybridization. The bars show 100 μ m and 20 μ m in the main images and insets, respectively. B, Relative expression of LRP1 and CCL3 mRNA in CD11b+ cells isolated from tumors in mLRP1+ mice (light bar) and mLRP1- mice (dark bar). CD11b+ cells were treated with 100 ng/ml LPS or vehicle before measuring CCL3 mRNA (n=3). C, Conditioned medium was recovered from an equivalent number of CD11b+ cells, isolated from tumors in mLRP1+ and mLRP1- mice. The cells were treated with 100 ng/ml LPS and allowed to condition SFM for 24 h before performing immunoblot analysis.

Figure 6.

Tumors developed in mLRP1⁻ mice demonstrate increased angiogenesis. A, Orthotopic tumors developed in mLRP1⁺ and mLRP1⁻ mice were immunostained for CD31. CD31 immunopositivity was quantitated for each tumor and summarized in the bar graph (*, $p < 0.05$). B, CD31 immunopositivity was determined in subcutaneous tumors developed in mLRP1⁺ and mLRP1⁻ mice. C, CD11b⁺ cells were isolated from tumors in mLRP1⁺ and mLRP1⁻ mice. VEGF mRNA expression was determined by qPCR (ns, not statistically significant). D, VEGF mRNA expression was corrected to account for the density of TAMs in tumors developed in mLRP1⁺ and mLRP1⁻ mice.