Cell surface receptor FPR2 promotes anti-tumor host defense by limiting M2 polarization of macrophages

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Abstract

FPR2 (Fpr2 in mouse) is a G protein-coupled receptor interacting with bacterial and host-derived chemotactic agonists. Fpr2 supports innate and adaptive immune responses as illustrated by the reduction in severity of allergic airway inflammation in Fpr2-KO mice, due to impaired trafficking of antigen presenting dendritic cells (DCs). The aim of this study is to examine the role of Fpr2 in host anti-tumor responses. We found that Fpr2-KO mice bearing subcutaneously implanted Lewis lung carcinoma (LLC) cells exhibited significantly shortened survival than normal mice due to more rapidly growing tumors. In contrast, in Fpr2-transgenic mice over-expressing Fpr2, subcutaneously implanted LLC tumors grew more slowly than those in wild type (WT) littermates. Investigation of tumor tissues revealed an increased number of macrophages associated with tumors grown in Fpr2-KO mice. Macrophages derived from Fpr2-KO mice showed a more potent chemotactic response to LLC-derived supernatant (LLC Sup), which could be neutralized by an anti-CCL2 antibody. The increased chemotaxis of Fpr2-KO mouse macrophages in response to LLC Sup was due to their higher level expression of CCR4, a chemokine receptor that also recognizes the ligand CCL2. Furthermore, macrophages from Fpr2-KO mice acquired an M2 phenotype after stimulation with LLC Sup. These results suggest that Fpr2 plays an important role in host defense against implanted LLC by sustaining macrophages in an M1 phenotype with more potent anti-tumor activities.
Introduction

A hallmark of cancer-associated inflammation is the infiltration of leukocytes, particularly cells of the myeloid lineage during tumor development (1). Tumor-infiltrating leukocytes are believed to be recruited by chemokines and the cells in turn promote tumor growth by producing growth-inducting and angiogenic factors (2). Macrophages are a major tumor-infiltrating cell type that can either impede or promote tumor progression (3). Blood-derived monocytes enter tumor tissues and differentiate into macrophages followed by further development into M1 or M2 cells, which differ in their patterns of cytokine secretion and function (1). The “classically-activated” M1 macrophages contribute to tumor rejection through type I cytokine production and antigen presentation (3, 4). The “alternatively-activated” M2 macrophages enhance angiogenesis and tissue remodeling through type II cytokines (5, 6). In more than 80% of cancers, tumor-associated macrophages (TAMs) mostly possess an M2 phenotype (7). Tumor- and stroma-produced mediators, including a variety of chemokines, promote the recruitment and activation of TAMs, which stimulate tumor cell proliferation, migration, angiogenesis and metastasis (8). Experimental and clinical studies have shown that the chemokine, monocyte chemoattractant peptide-1 (MCP-1, also known as CCL2), is most frequently expressed by tumor cells and its concentration is correlated with the degree of macrophage infiltration in tumors (7, 9).

CCL2 was initially found to interact with a G protein-coupled receptor (GPCR) CCR2 on macrophages which express a plethora of chemoattractant GPCRs (10). Subsequently, CCL2 was found to also activate CCR4, a promiscuous chemokine GPCR that is
expressed in Th2 lymphocytes (10, 11). In addition to chemokine GPCRs, macrophages also express classical chemoattractant GPCRs including formyl peptide receptors (FPRs) originally identified as receptors for bacterial chemotactic peptides (12, 13). FPRs, a family of structurally and functionally related GPCRs, are considered as a type of pattern recognition receptors (PRRs) due to their unique feature of sensing both pathogen and host derived danger signals (14). In mouse, at least two functional Fprs (Fpr1 and Fpr2) have been identified. Mice deficient in Fpr1 are more susceptible to infection by *Listeria monocytogenes* (15). Depletion of Fpr2 revealed that the receptor participates in innate and adaptive immune responses as shown by reduced severity of allergic airway inflammation and defective dendritic cell trafficking (16). Thus, Fpr2-KO mice clearly have compromised immune systems. We therefore hypothesized that Fpr2 may play a broad role in mediating inflammatory and immune responses including host defense against tumor progression. The aim of this study is to clarify the involvement of Fpr2 in anti-tumor host responses. Our observations demonstrate that Fpr2 deficiency results in the increased infiltration of macrophages in response to tumor-derived chemokine CCL2 and polarization of the macrophages into an M2 phenotype to support tumor progression.
Materials and Methods

Animals

The generation of Fpr2-KO mice was previously detailed (16). Fpr2-KO mice were backcrossed for at least eight generations to WT C57BL/6 mice before use. Fpr2-Tg were generated with human β-actin promoter on an FVB background and backcrossed to a C57BL/6 background for at least eight generations. Male mice of 8-10 wk old were used. Animal care was provided in accordance with the procedures outlined in the “Guide for Care and Use of Laboratory Animals” (National Research Council; 1996; National Academy Press, Washington D.C.).

Cell culture

Tumor cell lines used in this study include mouse Lewis lung carcinoma (LLC) cells and B16 melanoma cells, which were obtained from American Type Culture Collection (ATCC) and maintained in National Cancer Institute DCTD Tumor Repository. The NT2.5 mouse mammary tumor cell line was kindly provided by Dr. Elizabeth Jaffee of the Johns Hopkins University. All cell lines were tested for their mouse origin by using the Molecular Testing of Biological Materials assays by Animal Health Diagnostic Laboratory at National Cancer Institute-Frederick in 2009. All cell lines were cultured in DMEM containing 10% fetal calf serum (FCS). For collecting supernatants, the cells grown in 10% FCS media for 2 days were cultured in DMEM containing 2% FCS for 1 day. The supernatants were collected and centrifuged to remove debris and kept at -20°C before use.
Macrophage differentiation

Bone marrow (BM) cells were harvested from mouse femurs. Red cells were depleted by ACK lysis (Lonza). The remaining cells were washed once by PBS and suspended in DMEM containing 20% FCS and 20 ng/mL murine M-CSF (Peprotech). After 3 days, half of the medium was replaced with fresh medium. On Day 4, the cells containing more than 80% CD11b+ /F4/80+ macrophages were collected and analyzed.

Tumor implantation

LLC cells (5 × 10^5) in 100 µL PBS were sc injected into the mouse right flank. The tumor size was monitored twice a week and tumor volume was calculated as follows: Volume (mm³) = 0.5 × length × width × width. On Day 15, mice were euthanized and tumors were harvested for frozen section. In survival experiments, mice were euthanized when tumors exceeded 2 cm in diameter or contained visible necrosis. In metastasis experiments, 4 × 10^5 LLC cells in 250 µL PBS were iv injected into the mouse tail vein. On Day 15, the lungs were harvested and submerged into Bouin’s solution (Sigma-Aldrich) for counting tumor nodules. Mouse experiments were performed in accordance with the procedures outlined in the “Guide for Care and Use of Laboratory Animals” (National Research Council; 1996; National Academy Press, Washington D.C.).

Immunofluorescence staining

Cryosections of LLC tumors were stained with anti-CD11b (eBioscience), anti-Ly6G (eBioscience), anti-F4/80 (eBioscience), or anti-CD31 (eBioscience) antibody respectively, followed by a biotinylated secondary antibody (Santac Cruz) and
streptavidin-FITC with DAPI (Invitrogen) counterstaining to detect tumor-infiltrating myeloid cells and vasculature. Cryosections were also stained with an anti-iNOS antibody (Spring Bioscience) followed by a FITC-labeled secondary antibody (Santa Cruz) with DAPI counterstaining. The fluorescence images were taken by a Zeiss confocal microscope (Carl Zeiss) or an Olympus IX71 microscope (Olympus).

Chemotaxis assay

Chemotaxis was measured with 48-well chemotaxis chambers (Neuro Probe Inc.). CCL2 (Peprotech) and LLC Sup were diluted with assay medium (RPMI 1640 containing 1% bovine serum albumin [BSA]). Macrophages were pre-treated with or without 2 μg/mL anti-CCR2 antibody (Abcam) or 500 nM CCR4 antagonist (sc-221406, Santa Cruz) at 37°C for 1 hour and then resuspended in assay medium before being loaded into the upper compartment of the chemotaxis chamber. For neutralizing CCL2, the medium containing CCL2 or LLC Sup was incubated with 1 μg/mL anti-CCL2 antibody (R&D) at 37°C for 30 minutes before adding to the lower compartment of the chemotaxis chamber. Two compartments were separated by an 8-μm pore size polycarbonate membrane (GE Osmonica Labstore). After incubation for 1.5 hour at 37°C, cells that migrated across the membrane were stained and counted under light microscopy. The results were expressed as the mean (± SD) of migrating cells in 3 high powered fields (HPF) or chemotaxis index representing the fold increase in the number of migrated cells in response to chemoattractants over spontaneous cell migration in assay medium.

The effect of Fpr2 antagonists on macrophage polarization in vitro
BM-derived macrophages were pretreated with the Fpr2 antagonist WRW4 (4 µM for 36 hours) or LPG (5 µg/mL, 24 hours) and then were measured for chemotaxis induced by the Fpr2 agonist peptide MMK-1 or LLC Sup. BM-derived macrophages pretreated with Fpr2 antagonist LPG were also examined for expression of CCR4+ cells and Agr1 by Western blotting.

**Western blotting**

BM-derived macrophages treated with LLC Sup, LPS (1 µg/mL, Invivogen) plus IFNγ (10 ng/mL, Peprotech) or IL4 (20 ng/mL, Peprotech) plus IL13 (50 ng/mL, Peprotech) for different times were lysed. Total protein (10 µg) was subjected to 10% SDS-PAGE gel electrophoresis (Invitrogen) followed by transfer to PVDF membranes (Milipore), which were then treated with antibodies against Arg1 (BD), STAT1 (Cell Signaling Technology), phospho STAT1 (BD), STAT3 (BD), phospho STAT3 (Cell Signaling Technology), or phospho STAT6 (Calbiochem), respectively, and HRP-conjugated secondary antibody (Cell Signaling Technology).

**Isolation of tumor-infiltrating leukocytes**

Tumors were homogenized and total cells were centrifuged with discontinuous Percoll (Sigma-Aldrich) gradient (40% and 80%). Leukocytes were collected from the interface for further analysis.

**Flow cytometry**
BM cells and BM-derived macrophages were stained with antibodies against CD11b-PECy5, F4/80-FITC (eBioscience), and CCR4-PE (Biolegend). TAMs were labeled with CD45-FITC, CD11b-PE, F4/80-PerCP-Cy5.5 (eBioscience) and CD206-APC or CD11c-APC (Biolegend) antibodies. More than $10^5$ events were analyzed using a FACScan flow cytometer (BD) with CellQuest software (BD).

**RT-qPCR**

RT-qPCR procedures and primers are described in **Supplementary Table S1**.

**Statistical Analysis**

All experiments were performed at least 3 times with reproducible results. Data shown were from representative experiments. The statistical significance of differences between testing and control experimental groups was analyzed by $t$ test with Graphpad Prism Software (WattMaster Controls). Mouse survival was analyzed by Log-rank (Mantel-Cox) test with Graphpad Prism Software.
Results

Decreased survival of Fpr2-KO mice following LLC implantation

Since Fpr2 plays a major role in innate and adaptive immune responses (16), we first examined the role of Fpr2 in mouse resistance to LLC tumors. After sc injection of LLC cells, tumors formed in Fpr2-KO mice grew more rapidly than tumors formed in WT littermates (Fig. 1A). Fig. 1B shows that the survival rate of Fpr2-KO mice bearing LLC tumors was significantly reduced as compared with WT mice. Moreover, in a tumor metastasis model, after iv injection of LLC cells, more tumor nodules were found in the lung of Fpr2-KO mice (Fig. 1C) with shortened survival as compared to WT mice bearing lung LLC tumor nodules (Fig. 1D). We also found that LLC tumors subcutaneously implanted in Fpr2-Tg mice grew more slowly than the tumors in WT mice (Fig. 1E) with an increased survival rate of Fpr2-Tg mice (Fig. 1F). These results indicate that Fpr2 plays an important role in promoting host anti-tumor responses.

Increased myeloid cell infiltration and angiogenesis in LLC tumors formed in Fpr2-KO mice

Since leukocytes infiltrate a variety of tumors as a host response, we next examined myeloid cell infiltration in tumors grown in Fpr2-KO mice. The infiltration of CD11b+ myeloid cells was significantly increased in LLC tumors formed in Fpr2-KO mice than in the tumors grown in WT mice (Fig. 2A). We also detected an increased number of Ly6G+ granulocytes, which included myeloid-derived suppressor cells and neutrophils, in tumors in Fpr2-KO mice (Fig. 2B). Additionally, we detected increased F4/80+ TAMs in tumors formed in Fpr2-KO mice as compared with WT mouse tumors (Fig. 2C).
Moreover, the number of vasculature stained by CD31 in tumors was markedly increased in Fpr2-KO mice (Fig. 2D). In contrast, the number of vasculature in tumors formed in Fpr2-Tg mice was reduced as compared with WT mice (data not shown). Thus, the infiltration of myeloid cells, in particular TAMs, is increased in tumors formed in Fpr2-KO mice, which is associated with more rapid tumor growth and vigorous angiogenesis.

We further analyzed CD11b+Gr-1\textsuperscript{high} and CD11b+Gr-1\textsuperscript{low} cell populations in the mouse blood and spleen. There was no difference in both cell populations in the spleen between WT and Fpr2-KO mice regardless of tumor growth. However, in the blood of Fpr2-KO mice bearing LLC tumors, the CD11b+Gr-1\textsuperscript{low} cell population was significantly increased as compared to WT mice (Supplementary Table S2), suggesting an expansion of a “suppressive” myeloid cell population in the circulation of Fpr2-KO mice in response to tumor implantation.

*Increased chemotactic response of Fpr2-KO mouse macrophages to LLC tumor cell supernatant (Sup) and CCL2*

Since there were more TAMs in LLC tumors formed in Fpr2-KO mice, we hypothesized that the tumor microenvironment may produce factors that more actively attract Fpr2-KO mouse macrophages. To test this hypothesis, we differentiated macrophages from mouse bone marrow (BM) cells with M-CSF *in vitro* and examined their chemotactic responses to LLC Sup. **Figure 3A** shows LLC Sup induced a more potent migratory response of BM macrophages from Fpr2-KO mice than the cells from WT mice. In parallel experiments, macrophages from Fpr2-KO mice also showed an increased chemotactic
response to the chemokine CCL2. In contrast, BM-macrophages from Fpr2-Tg mice showed reduced chemotaxis toward LLC sup or CCL2 than macrophages from WT mice (Fig. 3B). We also tested the migration of macrophages from WT and Fpr2-KO mice in response to other chemoattractants that do not use CCR2. No significant difference was found in cell responses to LTB4 and C5a (Supplementary Fig. S1), suggesting that the change in macrophage responses to CCL2 after Fpr2 deletion was selective. These results indicate that Fpr2 deficiency renders macrophages more responsive to LLC Sup or CCL2, which may result in increased recruitment of TAMs.

To identify the chemotactic factors released by LLC cells, we utilized neutralizing antibodies against chemokines that have been implicated as macrophage chemoattractants in a tumor microenvironment. The chemotactic capability of LLC Sup for macrophages was completely abrogated by an anti-CCL2 antibody (Fig. 3C), which is not cross-reactive with other macrophage chemoattractants such as C5a (Supplementary Fig. S2), indicating that CCL2 is the major macrophage chemoattractant released by LLC cells. Since CCR2 is the major receptor for CCL2 (10), we examined whether macrophages only utilized CCR2 for migration in response to LLC Sup. Fig. 3C shows that anti-CCR2 antibody partially inhibited the chemotaxis of BM-derived macrophages induced by LLC Sup as well as by CCL2, suggesting that CCR2 is involved but receptors other than CCR2 may also participate in macrophage chemotaxis in response to LLC Sup.

*Increased expression of CCR4 by Fpr2-KO mouse macrophages*
Since CCL2 interacts with multiple receptors expressed by various leukocyte populations (17), we examined the expression of other chemokine receptors interacting with CCL2 by macrophages from both WT and Fpr2-KO mice. Mouse BM-derived CD11b⁺ myeloid cells expressed another CCL2 receptor CCR4, which is implicated in mediating Th2 immune responses (10, 11) and BM cells from Fpr2-KO mice showed an increased CD11b⁺CCR4⁺ population (Supplementary Fig. S3). The level of CCR4 expression was also increased on Fpr2-KO mouse macrophages as compared to the cells from WT mice (Fig. 4A). In contrast, CCR4 expression was reduced on macrophages of Fpr2-Tg mice (Fig. 4B). We then tested the contribution of CCR4 to the chemotaxis response of macrophages from Fpr2-KO mice to LLC Sup. Figure 4C shows that a CCR4 antagonist inhibited the response of Fpr2-KO mouse macrophages to LLC Sup by 50%. Treatment of macrophages with both anti-CCR2 antibody and CCR4 antagonist completely abolished the cell migration in response to LLC Sup and CCL2 (Fig. 4D).

We additionally measured CCR4 ligands such as CCL 17 and CCL 22 in LLC Sup and detected only CCL2 (Supplementary Fig. S4) (11). This is consistent with our findings that an anti-CCL2 antibody completely inhibited macrophage chemotactic activity of LLC Sup (Fig. 3C). Furthermore, we detected CCL2, but not other CCR4 ligands, released by LLC tumor cells derived from freshly dissected tumors from mice (Supplementary Fig. S5). To examine whether CCR4 was selectively increased in Fpr2 KO mouse macrophages, we measured the expression of several chemokine receptors. The expression of CCR1, CCR5 and CXCR4 was similar in naïve BM-derived macrophages or TAMs from WT and Fpr2-KO mice (Supplementary Fig. S6-7),
supporting our observation that CCR4 was an important player in cooperation with CCR2 in promoting TAM accumulation and LLC tumor growth in Fpr2-KO mice. Thus, CCR4 contributes to the more potent chemotactic response of Fpr2-KO mouse macrophages in response to LLC Sup and CCL2.

**Decreased expression of M1 markers by Fpr2-KO macrophages in response to LLC Sup**

Having observed that macrophages from Fpr2-KO mice more potently migrated in response to the LLC Sup, we next examined the capacity of LLC cells to affect the phenotype of macrophages. After incubation with LLC Sup, the expression of TNFα mRNA was up-regulated in WT, but not in Fpr2-KO, mouse macrophages (Fig. 5A). LLC Sup also significantly up-regulated iNOS mRNA in WT, but not in Fpr2-KO mouse macrophages (Fig. 5B). Immunostaining revealed that iNOS protein was markedly reduced in LLC tumors formed in Fpr2-KO mice as compared to tumors formed in WT mice (Fig. 5C and Supplementary Fig. S8A). Since TNFα and iNOS are M1 markers, our results suggest that Fpr2-KO mouse macrophages are defective in developing an M1 phenotype in response to tumor-released factors.

Further examination of M1 polarization of macrophages showed that LPS/IFNγ markedly up-regulated M1 markers TNFα and iNOS in WT, but not in Fpr2-KO mouse macrophages (Fig. 5D and Supplementary Fig. S8B-C). Another M1-associated molecule, STAT1, which is activated by LPS and IFNγ (5), was also rapidly phosphorylated in WT mouse macrophages upon LPS/IFNγ stimulation. In contrast, LPS/IFNγ-stimulated phosphorylation of STAT1 was at a lower level in Fpr2-KO mouse
macrophages (Fig. 5E-F). However, STAT1 phosphorylation was increased in Fpr2-Tg mouse macrophages as compared to WT mouse cells (Fig. 5G-H). These results confirm the importance of Fpr2 in maintaining macrophages in an M1 phenotype.

Increased expression of M2 markers by Fpr2-KO mouse macrophages in response to LLC Sup

We then examined the expression of an M2 marker Arg1 by macrophages in response to LLC Sup. Although the Arg1 mRNA expression was increased in both WT and Fpr2-KO mouse macrophages upon LLC Sup stimulation (Fig. 6A), Arg1 was more potently up-regulated in Fpr2-KO mouse macrophages. This was corroborated by a markedly higher level of Arg1 protein detected in Fpr2-KO mouse macrophages upon LLC Sup treatment (Fig. 6B-C).

We also examined Arg1 mRNA in macrophages under M2 polarization with IL4/IL13 stimulation (Fig. 6D) and found that Arg1 mRNA was more potently up-regulated in Fpr2-KO mouse macrophages compared to WT cells. We additionally examined the phosphorylation of STAT3/STAT6, markers for M2 (5), in mouse macrophages stimulated by IL4/IL13. Figure 6E-G shows that IL4/IL13 induced a more potent STAT3/STAT6 phosphorylation in Fpr2-KO mouse macrophages as compared to WT mouse cells. Thus, Fpr2-KO mouse macrophages exhibit an M2 polarized phenotype in response to LLC Sup as well as to type II cytokines.

Increased M2 polarization of TAMs in LLC tumors in Fpr2-KO mice
We then investigated the polarization state of TAMs in LLC tumors. The number and proportion of tumor-infiltrating leukocytes were significantly increased in tumors grown in Fpr2-KO mice for 14 days (Fig. 7A-B). Moreover, the number and proportion of CD206^+ M2 (18) macrophages were increased with reduced CD11c^+ M1 macrophages in LLC tumors grown in mFpr2-KO mice as compared with tumors from WT mice (Fig. 7C).

The effect of Fpr2 antagonists on macrophage phenotype

Based on enhanced M2 polarization in vitro and in macrophages of tumors from Fpr2-KO mice, we hypothesized that the presence of Fpr2 may support macrophage polarization into M1 phenotype by interacting with potential endogenous ligands. We addressed this issue by inhibiting Fpr2 in WT mouse macrophages and then evaluating cell phenotype. Pretreatment of WT mouse macrophages with an Fpr2 antagonist peptide WRW4 resulted in increased cell chemotaxis to CCL2 and LLC sup. This was associated with an increased expression of CCR4 by macrophages (Supplementary Fig. S9A-B). Treatment of WT macrophages with another Fpr2 antagonist LPG increased the expression of Arg1 after stimulation with LLC Sup (Supplementary Fig. S9C-D). These results indicate that Fpr2 has the capacity to support M1 polarization of macrophages presumably by interacting with potential agonists in the media.
Discussion

In this study, we have shown that Fpr2 plays an important role in retaining anti-tumor host defense by limiting macrophage recruitment into the tumor in response to the chemokine CCL2 and their subsequent conversion into M2 phenotype.

CCL2 is the most frequently expressed tumor cell-associated chemokine. Inhibition of CCL2 reduces the infiltration of many cell types associated with tumors including macrophages (19), T lymphocytes (20) and carcinoma-associated fibroblasts (CAF) (21). Enhanced CCL2 expression in inflammatory colitis results in the infiltration of macrophages, which are a crucial mediator of colon carcinogenesis (19). CCL2 antagonists not only decreased intra-colonic macrophage infiltration, but also attenuated neovascularization, and reduced tumor growth (19). In our study, we found that CCL2 was the major chemokine secreted by LLC cells and plays a pivotal role in the recruitment of TAMs in LLC tumors. In addition, CCL2 has been described to promote the polarization of TAMs since CCL2 blockade leads to the skewing of TAM phenotype towards M1 in the host (22). Moreover, CCL2 produced by both tumor cells and the stroma participates in tumor metastasis by recruiting macrophages into metastatic tumors (9). Thus, CCL2 acts as a bridge of tumor and host macrophage interaction that favors tumor progression.

Since CCL2 is the major macrophage chemoattractant released by LLC cells, the canonical CCL2 receptor CCR2 was considered as a plausible candidate for mediating macrophage chemotaxis. Recently, it is reported that the recruitment of tumor-promoting
spleen-derived TAMs required signaling of CCR2 (23). However, in our study an anti-CCR2 antibody only partially inhibited the chemotaxis of BM-derived macrophages induced by LLC Sup as well as by CCL2. This led to the hypothesis that other chemokine receptors may be utilized by macrophages derived from Fpr2-KO mice to mediate chemotaxis in response to CCL2 contained in LLC Sup. One of the chemokine receptors also utilized by CCL2 is CCR4, which is not induced on T cells by anti-CD3/anti-CD28 in the Th2 polarization environment (24). CCR4 has also been implicated in the progression of solid tumors, such as gastric and breast cancer, as well as in promoting lung cancer metastasis (25, 26). In our study, CCR4 was increased on Fpr2-KO mouse macrophages as compared to the cells from WT mice. Moreover, treatment of macrophages with both anti-CCR2 antibody and CCR4 antagonist completely abolished cell migration in response to LLC Sup, indicating that both CCR2 and CCR4 are required. Therefore, CCR4 is responsible for the more potent chemotaxis of Fpr2-KO mouse macrophages in response to LLC Sup. CCL17 and CCL22 are also CCR4 (11, 27-29) ligands with unclear pathophysiological significance (10, 30), but we failed to detect these chemokines as products of LLC cells. Therefore, our study reveals a novel role for CCL2 and CCR4 in macrophage recruitment in favor of tumor progression.

Our study also indicates an important role of Fpr2 in controlling macrophage recruitment into tumor microenvironment where the cells are exposed to tumor-derived factors to differentiate into M2-like cells. Accumulating evidence shows that Fpr2 and its human counterpart FPR2 are involved in a broad spectrum of pathophysiologic processes. Recently, FPR2 (Fpr2) expressed in human and mouse neutrophils was reported to
interact with a host-derived ligand serum amyloid A \((31)\) produced by liver and melanoma cells to promote the secretion of the anti-inflammatory cytokine IL-10, which increases the interaction of neutrophils with invariant natural killer (NK) T cells to enhance the anti-melanoma host responses by limiting the immunosuppressive activity of neutrophils \((32)\). FPR2 (Fpr2) also guides the recruitment of mesenchymal stem cells into tumors in response to tumor-derived agonist LL-37 that is critical for the formation of tumor vasculature \((33)\). In addition, some endogenous FPR2 agonists are capable of promoting the differentiation of macrophages into M1 phenotype that exhibits anti-hepatoma activity \((34)\). We therefore postulated that Fpr2-deficient macrophages may become more refractory to M1 stimulating factors present in the tumor microenvironment. In fact, our data indicates that Fpr2-KO mouse macrophages are defective in developing an M1 phenotype but exhibit an M2-dominated phenotype in response to LLC Sup as well as to type II cytokine stimulation. Consistent with the immune deficiency shown by Fpr2 deficient mice \((19)\), our study suggests that Fpr2 directly or indirectly retains the immune competence of macrophages that is important for limiting tumor progression. Interestingly, treatment with Fpr2 antagonists increased the chemotactic response of WT mouse macrophages to CCL2, in association with increased expression of CCR4 and LLC Sup-stimulated Arg1. However, tumor microenvironment is complicated and it is likely that elevated CCR4 enables Fpr2 deficient macrophages to more actively accumulate at the tumor sites. The precise mechanisms by which Fpr2 supports M1 polarization requires further investigation.
It should be noted that in two additional mouse tumor models, we did not detect significant differences in tumor growth in Fpr2-KO or Tg mice as compared with WT mice (data not shown). We then found that these two tumor cell lines did not release any CCL2 or other CCR4 chemokine ligands, strongly suggesting a key role of CCL2 in chemoattracting TAMs and the ability of Fpr2 to uniquely limit macrophage responses to CCL2 and to polarize into an M2 phenotype. Therefore, tumors of different origin are heterogeneous in interaction with the host based on their ability to release factors into macro- and micro-environment to elicit specific host responses, which may be critical for the consideration of “individualized” therapies. Further studies with additional CCL2 releasing mouse tumors and human cancers are warranted. We nevertheless provided in this study a novel mechanistic insight into the role of Fpr2 in the host defense against LLC tumors and suggest Fpr2 as a potential target for enhancing anti-tumor host responses.
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References

Figure Legends

Fig. 1 Survival of mice following LLC implantation. (A) The growth of LLC tumors in WT and Fpr2-KO mice. $5 \times 10^5$ LLC tumor cells in 100 µL PBS were subcutaneously (sc) injected into the right flank of each mouse. On Day 15 after injection, tumor size was measured. *$p < 0.05$, significantly increased tumor growth in Fpr2-KO mice compared to WT mice. (B) The survival rate of mice bearing sc implanted LLC. *$p < 0.05$, significantly shortened survival rate of Fpr2-KO mice compared to WT mice. (C) The lung metastasis in WT and Fpr2-KO mice. $4 \times 10^5$ tumor cells in 250 µL PBS were iv injected into mouse tails. After 15 days, mice were euthanized and lung nodules were counted. *$p < 0.05$, significantly increased lung LLC nodules in Fpr2-KO mice compared to WT mice. (D) The survival rate of mice bearing iv implanted LLC. *$p < 0.05$, significantly shortened survival rate of Fpr2-KO mice compared to WT mice. (E) The growth of LLC tumors in WT and Fpr2-Tg mice. $5 \times 10^5$ LLC tumor cells in 100 µL PBS were sc injected into the right flank of each mouse. Tumor size was measured on Day 15. *$p < 0.05$, significantly reduced tumor growth in Fpr2-Tg mice compared to WT mice. (F) The survival of mice bearing sc implanted LLC. *$p < 0.05$, significantly prolonged survival of Fpr2-Tg mice compared to WT mice.

Fig. 2 Increased macrophage infiltration and angiogenesis in LLC tumors formed in Fpr2-KO mice. On Day 15 after sc injection of LLC, tumors were removed and sectioned. (A) Myeloid cell infiltration in LLC tumors. Infiltrating myeloid cells were stained by an anti-CD11b antibody. Blue: DAPI; Green: CD11b. (B) Granulocyte
infiltration in LLC tumors. Infiltrating granulocytes were stained by an anti-Ly6G antibody. Blue: DAPI, Green: Ly6G. (C) Macrophage infiltration in LLC tumors. Infiltrating macrophages were stained by an anti-F4/80 antibody. Blue: DAPI; Green: F4/80. *p < 0.05, significantly increased myeloid cell, granulocyte and macrophage infiltration in tumors grown in Fpr2-KO mice as compared to WT mouse tumors (A-C, right panels). (D) Increased vasculature in Day15-tumors in Fpr2-KO mice. Vasculatures were stained by an anti-CD31 antibody. Blue: DAPI; Green: CD31. *p < 0.05, significantly increased CD31 staining in tumors grown in Fpr2-KO mice.

**Fig. 3 Increased chemotactic response of Fpr2-KO mouse macrophages to LLC Sup and CCL2.** (A-B) Chemotaxis of BM-derived macrophages in response to LLC Sup and CCL2. BM-derived macrophages from WT, Fpr2-KO or Fpr2-Tg mice were measured for chemotaxis in response to different dilutions of LLC Sup and CCL2. The results are expressed as the Mean (± SD) of chemotaxis index representing fold increase in cell migration in response to chemoattractants over medium control (BM). *p < 0.05, significantly increased chemotaxis of Fpr2-KO mouse macrophages (A) and significantly decrease response of Fpr2-Tg mouse macrophages (B) as compared to WT mouse macrophages. (C) CCL2 neutralization on macrophage chemotactic activity of LLC Sup. The chemoattractants were incubated with anti-CCL2 antibody (1 μg/mL) at 37°C for 30 minutes before chemotaxis assay. The results were expressed as the chemotaxis index. *p < 0.05, significantly reduced chemotactic activity of LLC Sup and CCL2 incubated with anti-CCL2. (D) CCR2 neutralization on the macrophage chemotaxis. Macrophages were incubated with anti-CCR2 antibody at 37°C for 1 hour before chemotaxis assay. The
results were expressed as the mean number (± SD) of migrating cells in 3 high powered fields (HPF). *p < 0.05, significantly reduced responses of the cells pre-incubated with anti-CCR2.

**Fig. 4 Increased expression of CCR4 by Fpr2-KO mouse macrophages.** (A-B) CCR4 expression by BM-derived macrophages in WT, Fpr2-KO and Fpr2-Tg mice. The percentage of CCR4+ cells in CD11b+F4/80+ BM-derived macrophages was shown. (C) CCR4 blockade on macrophage chemotaxis. Macrophages were incubated with the CCR4 antagonist (CCR4 Anta, 500 nM) at 37°C for 1 hour before chemotaxis assays. The results were expressed as the mean number (± SD) of migrating cells in 3 HPF. *p < 0.05, significantly reduced responses of the cells treated with anti-CCR2 antibody. (D) Neutralization of both CCR2 and CCR4 on macrophage chemotaxis induced by LLC Sup and CCL2. Macrophages were incubated with anti-CCR2 antibody (2 μg/mL) and CCR4 Anta (500 nM) at 37°C for 1 hour before chemotaxis assay. The results were expressed as the mean number (± SD) of migrating cells in 3 HPF. *p < 0.05, significantly reduced responses of the cells treated with anti-CCR2 and CCR4 Anta.

**Fig. 5 Decreased expression of M1 markers by Fpr2-KO macrophages in response to LLC Sup or type 1 cytokines.** (A) TNFα mRNA expression in BM-derived macrophages stimulated with LLC Sup. Total RNA of BM-derived macrophages was isolated 1 hour after LLC Sup (1: 5) stimulation then subjected to RT-qPCR to detect TNFα mRNA. *p < 0.05, significantly increased expression of TNFα mRNA by WT mouse macrophages as compared to Fpr2-KO mouse cells. (B) iNOS mRNA expression
in BM-derived macrophages. BM-derived macrophages were stimulated with LLC Sup (1: 5) for 6 hours. Total RNA was subjected to RT-qPCR to detect relative iNOS mRNA. *p < 0.05, significantly increased expression of iNOS mRNA by WT mouse macrophages as compared to Fpr2-KO mouse cells. (C) M1-like macrophage infiltration in LLC tumors. Infiltrating macrophages were stained by an anti-iNOS antibody. Blue: DAPI; Green: iNOS. Scale bar: 20 µm. (D) TNFα mRNA expression in BM-derived macrophages with LPS/IFNγ stimulation. Total RNA of BM-derived macrophages was isolated 12 hours after LPS (1 µg/mL) /IFNγ (10 ng/mL) stimulation then subjected to RT-qPCR to detect TNFα mRNA. *p < 0.01, significantly increased expression of TNFα mRNA by WT mouse macrophages as compared to Fpr2-KO mouse cells. (E-F) Decreased STAT1 phosphorylation in Fpr2-KO mouse macrophages upon LPS/IFNγ stimulation. After 4-day differentiation, BM-derived macrophages were stimulated with LPS/IFNγ. Cell lysate was subjected to Western blotting to detect phosphorylated STAT1. The relative intensity was normalized against total STAT1. *p < 0.01, significantly higher level of phosphor-STAT1 by WT mouse macrophages. (G-H) Increased STAT1 phosphorylation in Fpr2-Tg mouse macrophages stimulated with LPS/IFNγ. *p < 0.01, significantly increased STAT1 phosphorylation in Fpr2-Tg mouse macrophages as compared to WT mouse cells.

**Fig. 6 Increased expression of M2 markers by Fpr2-KO mouse macrophages in response to LLC Sup or type 2 cytokines.** (A) Arg1 mRNA expression in BM-derived macrophages. BM-derived macrophages were stimulated with LLC Sup (1: 5) for 6 hours. Total RNA was subjected to RT-qPCR to detect Arg1 mRNA. *p < 0.05, significantly
increased expression of Arg1 mRNA in Fpr2-KO mouse macrophages compared to WT mouse cells. (B-C) Arg1 protein levels in BM-derived macrophages. BM-derived macrophages were stimulated with LLC Sup (1:5) for different times. Cell lysate was subjected to Western blotting to detect Arg1. (D) Arg1 mRNA expression in BM-derived macrophages with IL4 (20 ng/mL)/IL13 (50 ng/mL) stimulation. Total RNA of macrophages was isolated 6 hours after IL4/IL13 stimulation then subjected to RT-qPCR to detect Arg1 mRNA. *p < 0.05, significantly increased expression of Arg1 mRNA in Fpr2-KO mouse macrophages compared to WT mouse cells. (E-G) Increased STAT3/STAT6 phosphorylation in Fpr2-KO mouse macrophages upon IL4/IL13 stimulation. BM-derived macrophages were stimulated with IL4/IL13 for different times. Cell lysate was subjected to Western blotting to detect phosphorylated STAT3/STAT6. *p < 0.05, significantly increased level of phosphorylated STAT3 in Fpr2-KO mouse macrophages compared to WT mouse cells. #p < 0.05, significantly increased level of phosphorylated STAT6 in Fpr2-KO mouse macrophages compared to WT mouse cells.

**Fig. 7 Increased M2 macrophage infiltration in LLC tumors grown in Fpr2-KO mice.** Total cells and leukocytes isolated from Day 14 mouse tumors were analyzed with FACS. Leukocytes are defined by CD45 staining. (A) The number of total cells and infiltrating leukocytes in tumors. *p < 0.01, significantly increased cell number in tumors grown in Fpr2-KO mice compared to WT mice. (B) The percentage of tumor-infiltrating leukocytes. *p < 0.05, significantly increased percentage of leukocytes in tumors grown in Fpr2-KO mice compared to WT mice. (C) The percentage of CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>CD206<sup>+</sup> and CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>CD11c<sup>+</sup> cells in tumor-
infiltrating leukocytes. *p < 0.05, significantly increased percentage of CD206⁺ cells in tumors of Fpr2-KO mice compared to WT mice. #p < 0.05, significantly decreased number of CD11c⁺ cells in tumors of Fpr2-KO mice compared to WT mice.
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**Liu et al. Figure 6**
Cell surface receptor FPR2 promotes anti-tumor host defense by limiting M2 polarization of macrophages

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