CCL5-Mediated Th2 Immune Polarization Promotes Metastasis in Luminal Breast Cancer

Qianfei Zhang1, Jilong Qin2, Lin Zhong3, Lei Gong3, Bing Zhang4, Yan Zhang1, and Wei-Qiang Gao1,5

Abstract

The tumor-promoting chemokine CCL5 has been implicated in malignant transformation of breast epithelial cells, with studies to date focusing mainly on basal-type breast cancers. In this study, we investigated the consequences of CCL5 deletion in the MMTV-PyMT transgenic mouse model of luminal breast cancer. In this model, primary tumor burden and pulmonary metastases were reduced significantly in CCL5-deficient subjects, an effect found to be associated with a deficit of Th2 (IL4+CD4+T) cells. Mechanistic investigations revealed that CCL5 activates CCR3, a highly expressed chemokine receptor on CD4+ T cells, and also boosts Gfi1 expression to promote the differentiation of Th2 cells, which enhance the prometastatic activity of tumor-associated myeloid cells. Clinically, polarization toward this immunosuppressive Th2 phenotype was also evident in patients with advanced luminal breast cancer. Thus, our findings showed that CCL5/CCR3 signaling promotes metastasis by inducing Th2 polarization of CD4+ T cells, with implications for prognosis and immunotherapy of luminal breast cancer.

Introduction

Luminal breast cancer is typically estrogen receptors (ER)+ and represents the largest proportion of women with breast cancer (around 70%; refs. 1, 2). Although luminal breast cancer patients respond to endocrine therapy, those with high ERα levels inevitably develop early and late relapses and metastasis. Discovery of new therapeutic targets for the treatment of these patients represents a big unmet medical need (1, 2).

CCL5/RANTES is one of the CC chemokine family proteins and interacts with G-protein–coupled receptors CCR1, CCR3, and CCR5 in various types of cells (3). Several studies have explored the association of CCL5 levels with breast cancer progression, and demonstrated that CCL5 expression is positively correlated with the disease stages at stages II and III (4, 5, 6). Our previous data also showed that host-derived CCL5 promotes breast cancer growth and metastasis by restraining the normal differentiation of MDSC subsets in a 4T1 mammary carcinoma model that mimics the triple-negative breast cancer (TNBC) in the clinic (7). However, whether and how CCL5 promotes progression and metastasis of luminal breast cancer has not been determined.

In this study, we investigated that roles of CCL5 in luminal breast cancer and found that CCL5 exerted its effects via CCR3 on CD4+ T cells and increased Gfi1 expression to induce the Th2 polarization of CD4+ T cells, which in turn promoted pulmonary metastasis of luminal breast cancer. In addition, there was a positive correlation between not only the tumor tissue expression levels of CCL5 (or IL4) or the serum CCL5 protein level (or peripheral blood Th2 cell numbers) and the progressiveness of clinical luminal breast cancer patients. These findings indicate that not only CCL5 can be a therapeutic target for the treatment of luminal breast cancer, but also serum CCL5 level and/or Th2 cell numbers in the peripheral blood might be new noninvasive prognostic indicators for monitoring the progression of luminal breast cancer.

Materials and Methods

Patients

Primary breast carcinomas were obtained from patients at the first affiliate hospital of Guangzhou Medical University (Guangzhou, China). Blood samples of 11 luminal breast cancer patients, 5 TNBC patients and 5 healthy human donors were gotten from Renji Hospital (Shanghai, China) and Yuhuangding Hospital (Yantai, China).

Mice

MMTV-PyMT in background of FVB/n and CCL5−/− mice in background of BALB/c were obtained from The Jackson Laboratory. To generate CCL5−/−PyMT mice, CCL5−/− mice were backcrossed into the FVB/n strain to N8, and then intercrossed with PyMT mice.

Study approval

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Shanghai Jiaotong University. All human samples were collected with the informed consent of the patients and the procedures were approved by Renji Hospital of Shanghai Jiaotong University (Renji [2013] No13).
Immune cell isolation

Lymph nodes and spleens were mechanically dissociated and strained through a 40-μm nylon mesh to produce a single cell suspension. After RBCs were lysed, CD4+ T cells were isolated with the CD4+ T-cell isolation Kit II (Miltenyi Biotec). For MDSCs, cells were stained with CD11b and Gr-1 antibodies, and then sorted by flow cytometry. For naive CD4+ T cells, cells were isolated with the CD4+CD62L+ T-cell isolation Kit II (Miltenyi Biotec). For tumor-infiltrating leukocytes (TIL), tumors were digested into single cells with collagenase type II (0.5 mg/mL), collagenase type IV (0.5 mg/mL), hyaluronidase (10 U/mL), and DNase I (0.01 mg/mL) for 2 hours at 37°C. The dissociated cells were collected, lysed by RBC lysis buffer, and then incubated with CD45 monoclonal antibody. For tumor-associated macrophages (TAM), the cells were incubated with F4/80 and CD11b monoclonal antibody. The positive cells were next sorted by flow cytometry on BD FACSAria using BD FACSDiva software. The purity of the isolated subpopulations regularly exceeded 90%.

Cell culture condition

A total of 1.5 × 10⁶ naïve CD4+ T cells were activated with 1.0 × 10⁷ T-depleted splenocytes as APC with 3 μg/mL anti-CD3 and 3 μg/mL anti-CD28 Abs. For Th2 differentiation the cells were cultured with 10 ng/mL IL4 and 10 μg/mL anti-IFNγ. For serum-induced differentiation of naïve CD4+ T cells, IL4 and anti-IFNγ were replaced by 50% V/V serum from MMTV-PyMT mice. The cells were differentiated for 1 week and the culture medium was changed every 2 days. For the study of signaling pathway, naïve CD4+ T cells from spleens of CCL5+/− and CCL5−/+ mice were stimulated with T-depleted splenocytes under Th2 condition for 3 days and culture with only IL2 for 1 day. Then cells were reactivated by T-cell–depleted splenocytes under Th2 condition for 1 hour (Erk/MAPK assay), then sorted by flow cytometry on BD FACSAria using BD FACSDiva software. The purity of the isolated subpopulations regularly exceeded 90%.

3D organotypic coculture

Mouse mammary epithelial cells (MEC) were isolated as previously described (8). Briefly, the fourth mammary fat pads from 12-week-old CCL5+/− PyMT or CCL5−/+ PyMT were harvested. Then tissues were minced finely and digested with Collagenase A 3.0 mg/mL (Roche) and Trypsin 1.5 mg/mL (Gibco). Three-dimensional (3D) organotypic cultures were established as previously described (9, 10), and medium containing 10% Matrigel was replaced every 4 days. Coculture with TAM or CD4+ T cells was established when stable organoid formed about 2 weeks later. Immune cells were overlaid in medium containing 2% Matrigel and the percentage of invasion acini was assessed 2 days later.

Statistical analysis

The Student t test was used to analyze the data. Results are given as mean ± SEM unless otherwise indicated. P values <0.05 were considered significant.

Results

CCL5 expression is positively correlated with disease progression in an MMTV-PyMT mouse model of luminal breast cancer

We used an MMTV-PyMT transgenic mouse line, a reliable model of human luminal breast cancer (11), to study the role of CCL5 in this major type of breast cancer. By 5 weeks, female carriers developed palpable mammary tumors that were multifocal and involved the entire mammary fat pad. Approximately 94% of tumor-bearing females developed pulmonary metastasis by 3 months of age (12, 13). CCL5 expression in the tumor site was examined by IHC at ages of 5, 11, and 18 weeks, respectively. As expected, CCL5 expression was increased progressively during breast cancer tumorigenesis in the MMTV-PyMT mice (Fig. 1A), similar to the pattern of human breast cancer progression in the clinic (14).

Deletion of CCL5 inhibits luminal breast tumorigenesis and pulmonary metastasis in the MMTV-PyMT mouse model

To determine the significance of CCL5 expression in breast cancer, we generated PyMT mice bearing homozygous null CCL5 (CCL5−/−/PyMT). Notably, primary mammary tumor latency was dramatically longer in CCL5−/−/PyMT compared with the control cohort CCL5+/+/PyMT mice (Fig. 1B). The tumor burden at an age of 11 weeks was significantly decreased due to the loss of CCL5 expression whereas at an age of 18 weeks such difference almost disappeared (Fig. 1C). However, the pulmonary metastasis of 18-week CCL5−/−/PyMT mice was dramatically attenuated in both the tumor burden in the lung and the number of metastatic foci (Fig. 1D–F). To further confirm the attenuated metastasis in CCL5−/−/PyMT, we also examined the circulating tumor cell numbers (CTC, cytokeratin+/CD45−) in the peripheral blood and found that the number of CTC was also decreased significantly (Fig. 1G). These data indicate that deletion of CCL5 inhibits not only luminal breast tumorigenesis but also the pulmonary metastasis.

Th2 cells mediate a metastasis advantage of CCL5 in luminal breast cancer

Because the reduced pulmonary metastatic phenotype in the CCL5−/−/PyMT is similar to that of PyMT/CD4+ T mice reported previously by the Lisa M. Coussens’s group (9), we determined whether CCL5 deficiency would affect the polarization of CD4+ T cells in the progression of mammary carcinoma. TILs were first evaluated by flow cytometry and IHC analysis, for CCL5 is well known as a chemokine to recruit leukocytes to the site of infection. As shown in Fig. 2A–C, there was no difference between the numbers and the composition of CD45+ cells infiltrating primary tumors of 18-week-old CCL5−/−/PyMT and CCL5+/+/PyMT mice.

Then we isolated CD4+ T cells from TILs and tumor-draining lymph nodes (LN) of 18-week-old CCL5−/−/PyMT and CCL5−/−/PyMT mice by magnetic microbeads, and examined the expression of specific transcriptional factors by RT-PCR. At the same time, these isolated CD4+ T cells were ex vivo activated by leukocyte activation cocktail for 18 hours, culture medium were collected and effector cytokines indicative of Th1, Th2, Th17, and Treg response were examined by ELISA. As shown in Fig. 2D, GATA3 (Th2) expression was dramatically decreased in CD4+ T cells from both TILs and LNs of CCL5−/−/PyMT mice, whereas other transcriptional factors examined had no significant changes between these two cohorts except for the elevated expression of T-bet (Th1) in LNs. These results were echoed by the expression of effector molecules of a different subtype CD4+ T cells [Fig. 2E], indicating that tumor-induced Th2 polarization of CD4+ T cells was inhibited in CCL5−/−/PyMT mice. To further confirm these results, CD4+ T cells from tumor-draining LNs were ex vivo activated by leukocyte activation cocktail for 4 hours. Then effector cytokine
expression was examined by intracellular flow cytometry and these analyses revealed that the percentage of IL4+ CD4+ T cells (Th2) was markedly decreased (Fig. 2F and G). In conclusion, CCL5 deficiency in MMTV-PyMT mice inhibited CD4+ T cells polarization to Th2 cells during the progression of mammary carcinoma.

To explore the role of CCL5-induced Th2 polarization on tumor latency and primary tumor burden, we checked IL4 expression of CD4+ T cells isolated from spleen, tumor-associated LN (or axillary LN), TIL and lung at age of 6, 11, and 18 weeks. Because the great differences on tumor latency, tumor burden, and pulmonary metastasis between CCL5+/+ PyMT and CCL5−/− PyMT mice, respectively, existed at those time points, and we also examined the number of CTC cells in peripheral blood to monitor the status of tumor metastasis at each time point. The results of qPCR showed that only at age of 18 weeks, the mRNA level of IL4 was strongly increased in all of the CD4+ T cells of CCL5+/+ PyMT mice, compared with CCL5−/− PyMT mice (Supplementary Fig. S1), suggesting that lower level of Th2 cells may contribute to the reduced pulmonary metastasis, but not the increased tumor latency and reduced primary tumor burden of CCL5−/− PyMT mice.

To further confirm that the altered polarization of CD4+ T cells contributes to the reduced lung metastasis in CCL5−/− PyMT mice, we depleted T-cell subsets in vivo during carcinogenesis of CCL5+/+ PyMT and CCL5−/− PyMT mice by i.p. injection of 250 μg anti-CD4 antibody (GK1.5) on days 85, 86, 89, and 92, respectively (the efficiency of CD4+ T cells depletion was shown in Supplementary Fig. S3A). As shown in Fig. 2H and I, blocking of CD4+ T cells resulted in a significantly reduced number of pulmonary metastatic foci in CCL5+/+ PyMT mice compared with the controls, and consequently the difference of the pulmonary metastasis induced by CCL5 deficiency disappeared. The findings showing that Th2 cells are the major subtype of CD4+ T cells in the tumor microenvironment of CCL5+/+ PyMT mice argue against a possible involvement of other effector lineage of CD4+ T cells (Fig. 2F and G). To provide further supporting evidence for this interpretation, we examined the number of CTCs by flow cytometry. Consistent with the pulmonary metastasis analysis, there was no significant statistical difference in the number of CTCs between CCL5+/+ PyMT and CCL5−/− PyMT mice after depletion of CD4+ T cells (Fig. 3J). Collectively, these data suggested that Th2 cells play a major role in CCL5-induced pulmonary metastasis of luminal breast cancer.

Tumor-associated myeloid cell phenotype induced by CCL5 is CD4+ T cell dependent

Previous studies reported that tumor-associated myeloid cells, including TAMs and MDSCs, promote tumor cell invasion, and metastasis, which can be modulated by communicating with Th2 cells (15, 16). To determine whether these myeloid cell phenotype and effector bioactivity are affected by CCL5 deficiency and what is the relationship with CD4+ cells, we isolated TAMs from primary mammary tumor or MDSCs from spleens of both CCL5+/+ PyMT and CCL5−/− PyMT mice and compared their expression levels of several cytokines indicative of macrophages polarization or epithelial cell invasion. As shown in Supplementary Fig. S2A, the levels of cytokines, TGFβ and EGF, were significantly lower in the TAMs of the CCL5−/− PyMT mice, as compared with that of the control mice. The same tendency was observed in MDSCs (Supplementary Fig. S2B and S2C). However, following the blocking of the CD4+ T cells by using CD4-neutralizing antibody, the distinctive expression of cytokines and growth factors in TAMs and MDSCs between CCL5+/+ PyMT and CCL5−/− PyMT mice became largely diminished (Supplementary Fig. S3B and S3C), together indicating that it is CD4+ T cells that mediate the effect of CCL5 on activities of tumor-associated myeloid cells in MMTV-PyMT mice.
CD4⁺ T cells from CCL5⁻/⁻ PyMT mice inhibit invasion of mammary carcinoma cells in 3D organoid cultures by regulating properties of tumor-associated myeloid cells

To explore the mechanism on how CD4⁺ T cells from CCL5⁻/⁻ PyMT mice inhibit tumor invasion, we first established an ex vivo 3D organotypic culture model with primary MECs derived from PyMT mice. As described previously (10), MECs were collected from 12-week-old CCL5⁺/⁺ or CCL5⁻/⁻ PyMT mice, placed in growth factor–reduced Matrigel and allowed to form stable organoids for 2 weeks and the effects of CD4⁺ T cells and tumor-associated myeloid cells from CCL5⁻/⁻ PyMT mice on MECs’ invasion were first examined in this 3D coculture system. To simplify the experiment, we only chose TAMs as a potential mediator of CD4⁺ T lymphocytes. After 3 days, TAMs from CCL5⁺/⁺ PyMT dramatically enhanced organoid disruption of either CCL5⁺/⁺ or CCL5⁻/⁻ MEC, whereas TAMs from CCL5⁻/⁻ PyMT had no significant effects on the invasive phenotype of MECs (Fig. 3A). Morphologically, the organoids cocultured with CCL5⁺/⁺ TAMs had more spindle-shaped cells (Fig. 3B). These data suggest that TAM from CCL5⁻/⁻ PyMT mice lost the proinvasive activity, whereas loss of CCL5 in MECs did not affect their invasive activity, compared with the control group.

To further confirm the regulatory function of CD4⁺ T cells on metastatic activity of tumor-associated myeloid cells, these cells isolated from tumor-draining LNs were cocultured with MECs.
organoids in the presence of CCL5+/+ or CCL5−/− TAMs. As expected, the percentage of the invasive organoids induced by either CCL5+/+ or CCL5−/− TAMs was significantly increased in the presence of CCL5+/+ CD4+ T cells and exogenous CCL5 protein could not mimic the function of CD4+ T cells (Fig. 3C and D). On the contrary, CD4+ T cells from CCL5−/− PyMT inhibited proinvasive activity of MECs mediated by CCL5+/+ TAMs, indicating that a prometastatic activity of TAMs is further regulated by the status of CD4+ T cells (Fig. 3C and D). More importantly, in the absence of TAMs, CD4+ T cells from either CCL5+/+ PyMT or CCL5−/− PyMT alone had no effect on the invasion of MEC organoids (Fig. 3C and D, close bar). These data suggest that CD4+ T cells from CCL5-null mice inhibit MEC organoid invasion through modulating activity of tumor-associated myeloid cells.

To further explore the mechanism on the lower invasive activity of MECs induced by CCL5−/− CD4+ T cells, we examined the expression of EMT (epithelial–mesenchymal transition)-related genes in MEC organoids, for EMT allows the polarized epithelial cancer cells to acquire the mesenchymal cell phenotype, such as enhanced migratory capacity and invasiveness (17). The results showed that the expression of E-cadherin was higher and the expressions of vimentin and Zeb1 were lower in MEC organoids that cocultured with CCL5−/− CD4+ T cells, compared with those with control cells (Fig. 3E; Supplementary Fig. S4). These results were further confirmed by qPCR data of cancer cells from 18-week-old mice (Supplementary Fig. S5), suggesting that the lower rate of conversion from epithelia to mesenchyme may contribute to antinvasive activity of CCL5−/− CD4+ T cells.

CCL5 promotes Th2 cell differentiation in vitro

To determine whether loss of Th2 cells in CCL5+/− PyMT mice was attributable to the reduced differentiation of Th2 cell in the absence of CCL5 protein, we examined Th2 polarization of CD4+ T cells under CCL5-null conditions in vitro. Western blotting revealed that lots of CCL5 protein is expressed in CD4+ T cells (Supplementary Fig. S6), suggesting that the autocrined CCL5 protein from T cells and feeder cells is sufficient for inducing Th2 polarization in vitro. Naïve CCL5−/− CD4+ T cells or wild-type controls were, therefore, activated under Th2 or Th0 conditions for 7 days, and cytokine phenotypes were examined. As shown in Fig. 4A and B, CCL5 deficiency dramatically inhibited the generation of IL4+ CD4+ T cells under Th2 conditions, whereas CCL5 protein alone could not induce naïve T cells to Th2 differentiation under Th0 conditions. To further test whether exogenous CCL5 can rescue the defect on Th2 cells polarization of CCL5-null CD4+ T cells, recombinant murine CCL5 protein was added back and the addition of CCL5 at a concentration of 20 ng/mL almost completely recovered the Th2 polarization of CCL5-null CD4+ T cells (Fig. 4A and B). Collectively, either endogenous or exogenous CCL5 protein could cooperate with both IL4- and IFN-γ-neutralizing antibody to facilitate the generation of Th2 cells.

To further explore the role of CCL5 on CD4+ T-cell differentiation in vivo, we induced the differentiation of naïve CD4+ T-cell with the serum from 18-week-old CCL5+/+ PyMT or CCL5−/− PyMT mice, instead of IL4 and anti-IFN-γ. Flow cytometry data showed that the percentage of Th2 cells was dramatically decreased in CCL5−/− CD4+ T cells, but those of other subsets were not significantly increased, although percentages of Th1 were slightly increased, compared with the control group (Fig. 4C and D). These results suggested that CCL5 mainly promotes the Th2 differentiation, but not other subsets of CD4+ T cells, in the mouse model of luminal breast cancer.

Th2 differentiation is promoted by CCL5-induced Gfi1 expression

To figure out the mechanism underlying CCL5-induced Th2 differentiation of CD4+ T-cells, we suspected growth factor independent-1 (Gfi1), a zinc-finger transcriptional repressor (18), as a possible molecule involved in this process, because the phenotype of Gfi1-knockout mouse is similar to that of CCL5 null mice bearing 4T1 mammary carcinoma, that is, generation of atypical CD11b+Gr-1+ cells is induced by cytokines secreted by tumor cells (7, 19, 20). In addition, Gfi1 has been proven to play an essential role in CD4+ T cells polarization (21). To explore the
role of Gfi1 in CCL5-induced Th2 polarization, we first investigated the endogenous Gfi1 expression in CD4+ T cells. These assays showed that the endogenous Gfi1 and IL4 expression in CD4+ T cells isolated from the tumor-associated leukocytes were dramatically decreased in CCL5-null tumor-bearing mice (Fig. 5A).

Because Gfi1 can be induced by IL4 during Th2 polarization of CD4+ T cells (21), its induction in CCL5−/−/−CD4+ T cells was studied in detail. As shown in Fig. 5B, although there was not much difference between these two groups by 3 days of induction, the amount of Gfi1 protein in CCL5−/−/− cells was significantly lower than that of CCL5+/+ cells on day 7 (Fig. 5B; Supplementary Fig. S7A). To further confirm these results, rmCCL5 protein was added back to the cell culture medium of CCL5−/−/−CD4+ T cells at increased doses and the expression of Gfi1 protein was gradually increased to the comparable level of that in CCL5+/+ cells (Fig. 5C; Supplementary Fig. S7B). These results suggest that CCL5 might act as an upstream ligand to induce Gfi1 expression to promote Th2 polarization of CD4+ T cells.

To determine whether reintroduction of Gfi1 rescues the defect on Th2 polarization in CCL5−/− cells, we infected isolated naïve CCL5−/−/−CD4+ T cells with lentiviruses encoding Gfi1 and GFP and induced these cells to differentiate into Th2 cells.

Figure 4.

CCL5 enhances generation of Th2 cells in vitro. A and B, FACs analysis of IL4+/CD4+ T cells in CD4+ T cells after 7 days of Th2 polarization. Shown in B is the percentage of IL4-positive cells in A. C and D, IFNγ, IL4, FoxP3, and IL17 staining of CD4+ T cells. The protocol is same as A and B, except for replacing Th2-inducing cytokines and antibodies with sera from tumor-bearing mice. *, P < 0.05; **, P < 0.01.

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CCR3 is the functional receptor mediating CCL5-induced Th2 differentiation

It is well known that CCL5 induces leukocyte migration via three high-affinity receptors of the CCPR family, namely CCR1, CCR3, and CCR5 (23). Real-time RT-PCR revealed that expression of CCR3 was much higher in CD4+ T cells and TILs than that in
tumor cells (Supplementary Fig. S9). To further determine whether these three receptors are all involved in the pro-Th2 cell polarization activity of CCL5, we examined their expression pattern during Th2 differentiation in CD4⁺ T cells by RT-PCR. The transcript of CCR1 from naïve CCL5⁺/⁺ CD4⁺ T cells was set as 1 and others were shown in values relative to that of CCR1. As shown in Fig. 6A–C, all of transcripts for CCR1, CCR3, and CCR5 were almost undetectable in freshly isolated naïve CD4⁺ T cells. However, there were some differences in the subsequent days: The levels of mRNAs for CCR1 were barely detectable at days 3 and 7, whereas these for CCR3 were dramatically increased at day 3 and kept on at high levels until day 7. For CCR5, it became readily measurable at days 3 and 7, but the expression level was significantly lower compared with the transcript of CCR3. To further confirm the expression pattern of CCR3 and CCR5 on CD4⁺ T cells, we also examined their expression by immunofluorescence staining (Fig. 6D and E). These results were consistent with the qPCR data as well as the previous report showing that specific chemokine receptors are associated with polarized subsets of CD4⁺ T cells and that CCR3 is mainly expressed in Th2 polarization (24).

Next, we used inhibitors of chemokine receptors to determine which of these receptors is important for CCL5-induced Th2 differentiation. Naïve CD4⁺ T cells from WT mice were treated under the Th2 condition for 2 days before the addition of CCR3 or CCR5 inhibitor into the cell culture medium. Not surprisingly, the CCR3 inhibitor (SB328437) significantly suppressed the Th2 polarization of CD4⁺ T cells, whereas the CCR5 inhibitor (Maraviroc) had little effect on it (Fig. 6F and G).

Considering the fact that our culture system contained some APCs that might affect the Th2 polarization via secretion of IL12 (25), we performed an experiment by adding IL12p40-neutralizing antibody to the Th2-induction medium. As shown in Fig. 6F and G, inactivation of IL12 did not significantly alter CCL5-induced Th2 differentiation. These results suggest that CCL5 promotes Th2 polarization by directly activating CCR3 on CD4⁺ T cells, rather than via an indirect contribution from APCs.

High expression of CCL5 and IL4 is relevant to the aggressiveness of clinical luminal breast cancer

Previous studies showed that CCL5 expression is positively correlated with human breast cancer progression only at stages II and III (4, 5). To validate the correlation between increased expression levels of CCL5 and IL4 and the tumor grades of human luminal breast cancer samples, we took tissues from paraffin-embedded clinical samples of both normal and luminal breast tumors (stages II and III) and conducted a TaqMan qRT-PCR assay to measure their expression. We found that the expression of both CCL5 and IL4 were significantly higher in more aggressive tumors than less malignant tumors or the normal breast tissue (Fig. 7 A and B), and the correlation...
analysis revealed that expression levels of CCL5 and IL4 were positively correlated (Pearson’s $r = 0.75$; Fig. 7C). Furthermore, we observed a direct correlation between CCL5 and IL4 expressions in the breast cancer tissue by immunohistochemistry and both of them were positively associated with the malignant grades of breast cancer (Fig. 7D). Clinically, based on Her2 expression, ER$^+$ luminal breast cancer can be divided into two types: luminal A (Her2$^-$) and luminal B (Her2$^+$). So we grouped these breast cancer patients according to their molecular markers and examined expression levels of CCL5 and IL4 between these two groups (Supplementary Fig. S10A and S10B). The results showed that there was no discrimination between luminal A and luminal B types.

Serum CCL5 levels and numbers of peripheral blood Th2 cells are positively correlated with aggressiveness of human luminal breast cancer

To provide further evidence that CCL5 enhances Th2 polarization, we examine the correlation between CCL5 expression and the number of Th2 cells in the peripheral blood of luminal breast cancer patients. The results demonstrated that serum CCL5 levels and percentages of IL4$^+$ CD4$^+$ T cells in the peripheral blood were significantly higher in luminal breast cancer patients at stage III as compared with stage II (Fig. 7E and F; Supplementary Fig. S10C). The correlation analysis showed that serum CCL5 levels and the numbers of peripheral IL4$^+$CD4$^+$ T cells were positively correlated in malignant breast cancer patients (Pearson’s $r = 0.6229$; Fig. 7G).

Discussion

Previous studies mainly focused on roles of CCL5 in basal breast cancer and interpreted its role via different mechanisms. For example, CCL5 recruits the accumulation of deleterious TAMs in the mammary carcinoma site and promotes its expression of MMP9 to facilitate the disease progression (14). In addition to indirectly potentiating cancer development by regulating properties of immune cells, research from several laboratories has revealed that CCL5 regulates tumor growth and invasion through direct activation of CCR5 receptors on MECs. For instance, Karooub and colleagues (26) demonstrated that MSC-derived CCL5 promotes human breast cancer cell invasion and release of MMPs by binding to CCR5 directly on cancer cells in a xenograft breast cancer model. On the other hand, it is important to point out that most of these studies have been done in basal breast cancer models (2). The present study shows clear evidence that CCL5 also plays an important role in luminal breast tumorigenesis and metastasis via a different mechanism, that is CCL5 exerts its effects via induction of Th2 polarization, rather than via direct binding to the myeloid cells or the tumor cells. Therefore, the present work...
sheds new light on the role of CCL5 during tumorigenesis and metastasis of breast cancer, in particular, the luminal breast carcinomas.

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

Authors’ Contributions
Conception and design: Y. Zhang
Development of methodology: Y. Zhang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Zhong, L. Gong, B. Zhang, Y. Zhang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Q. Zhang, J. Qin, L. Zhong, L. Gong, B. Zhang, Y. Zhang, W.-Q. Gao
Writing, review, and/or revision of the manuscript: Q. Zhang, J. Qin, Y. Zhang, W.-Q. Gao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Zhang
Study supervision: Y. Zhang, W.-Q. Gao

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