

Breast Phyllodes Tumors Recruit and Repolarize Tumor-Associated Macrophages via Secreting CCL5 to Promote Malignant Progression, Which Can Be Inhibited by CCR5 Inhibition Therapy



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Abstract

Purpose: Malignant phyllodes tumor (PT) is a fast-progression neoplasm derived from periductal stromal cells of the breast, which currently still lack effective treatment strategies. Our previous studies showed that the high density of tumor-associated macrophages (TAM) plays an important role in the malignant progression of PTs. TAMs secreted large amount of CCL18 to promote myofibroblast differentiation and invasion via binding to its receptor PIPTNM3 on myofibroblasts. Herein, we investigate the mechanism of how TAMs are recruited and repolarized by PTs to drive the malignant progression.

Experimental Design: The cytokines secreted by PTs were identified by the cytokine array. The clinical and pathologic correlations of the cytokine with PTs were estimated with IHC. The mechanisms of the cytokine that recruited and polarized the macrophage were explored with a coculture model of primary PT cells and macrophages *in vitro* and *in vivo*. The patient-derived xenografts (PDX) of malignant PTs were used to evaluate the therapeutic effect of CCR5 inhibitor.

Results: A high level of malignant PT-secreted CCL5 correlated with poor outcome of PTs and could be an independent prognostic factor of PTs. CCL5 bound to its receptor, CCR5, on macrophages thus activated AKT signaling to recruit and repolarize TAMs. Subsequently, the TAMs released CCL18 to further promote the aggressive phenotype of malignant PTs by enhancing and maintaining the myofibroblast differentiation and invasion *in vitro* and *in vivo*. In a murine PDX model of human malignant PTs, the CCL5–CCR5 axis blocked by maraviroc, an FDA-proved CCR5 inhibitor, prevented recruitment of monocytes to the tumor and dramatically suppressed tumor growth.

Conclusions: Our findings indicate that malignant PTs recruit and repolarize TAMs through a CCL5–CCR5–driven signaling cascade. Thus, a positive feedback loop of CCL5–CCR5 and CCL18–PIPTNM3 between myofibroblast and TAMs is constituted to drive the malignant progression of PTs. Furthermore, targeting CCR5 with maraviroc represents a potential clinically available strategy to treat malignant PTs.

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Introduction

Breast phyllodes tumor (PT) is a biphasic tumor and constitutes about 1% of all breast tumors. Unlike the usual breast tumor that derives from the epithelium, PT is derived from the breast fibroepithelial tissues (1). PTs are histologically classified as benign, borderline, or malignant. Malignant PT is featured by frequent local relapse and distant metastasis and current adjuvant chemotherapy or radiotherapy is not effective against malignant PTs (2). Our previous studies have indicated myofibroblasts were the major malignant component of PTs, marked with high expression of α -smooth muscle actin (α -SMA; ref. 3). Currently, PTs are histologically classified on the basis of stromal cellularity, mitotic activity of stromal cells, stromal nuclear atypia, stromal overgrowth, and types of border (infiltrating or pushing). However, the clinical outcome of PT is hard to predict based on these histologic observations. Therefore, there is a pressing requirement to understand the mechanisms of how the aggressive phenotype of PTs is regulated. Upon tissue injury, fibroblasts are activated and differentiate into myofibroblasts. Macrophages are usually found in injured tissue and are in close proximity with collagen-producing myofibroblasts (4). Macrophages produce profibrotic mediators that directly activate fibroblasts, including TGF- β 1,

Translational Relevance

The tumor-associated macrophages (TAM) have been demonstrated to participate in cancer progression in various ways. Therefore, targeting TAMs has been reported as a promising strategy for cancer treatment. Our study revealed that TAMs were recruited by CCL5 secreted from the malignant phyllodes tumor (PT) cells and acted as a driving factor that interacted with malignant PT cells to induce tumorigenesis. Herein, we demonstrate that maraviroc, an FDA-approved CCR5 inhibitor, can block the recruitment and polarization of TAMs by malignant PT, with a therapeutic effect even better than doxorubicin. Because malignant PT is not sensitive to chemotherapies, this study represents a clinically available strategy for malignant PT treatment that substitutes chemotherapies.

platelet-derived growth factor (PDGF; ref. 3), and insulin-like growth factor 1. These factors stimulate the proliferation and collagen synthesis of myofibroblasts (5). In the tumor microenvironment, tumor-associated macrophages (TAM) are the most abundant cell type (6). TAMs affect many aspects of tumor cell biology and drive malignancy progression, including cancerous cell proliferation, angiogenesis, invasion, metastasis, immunosuppression, and chemotherapy/radiotherapy resistance (7, 8). Clinical and epidemiological studies have shown a strong correlation between the increased TAMs' density and poor prognosis in several types of cancer (9, 10), including breast cancer (4, 11). Our previous studies showed that increased density of TAMs correlated with the malignant progression of breast PT (3). We also revealed that TAMs were essential in driving myofibroblast differentiation (fibroblasts–myofibroblasts transition, FMT) in the malignant progression of PTs. TAMs secreted CCL18 and activated the NF- κ B/*miR*-21/*PTEN*/*AKT* axis in myofibroblasts via its receptor *PIPTNM3*, resulting in the malignant progressions of PTs (12).

On the other hand, TAMs are recruited from monocyte-derived macrophages (MDM) and/or tissue-resident macrophages and then polarized in the cancer microenvironment. TAM heterogeneity is the outcome of interactions between tumor-derived, tissue-specific, and developmental signals (13). However, the mechanisms of macrophage recruitments and TAM repolarization in PTs remain largely unknown. In this study, we revealed that malignant PT cells secreted high levels of CCL5, which activated the CCR5/*AKT* pathway of surrounding macrophages, therefore recruiting macrophages and inducing the phenotype changes of TAMs. Targeting CCR5 with clinical available inhibitor is a promising strategy to prevent the malignant progression of PTs.

Materials and Methods

Patient tissue specimens

Breast PT samples were obtained from 323 female patients with 199 benign, 46 borderline, and 78 malignant PTs at the Breast Tumor Center, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University (Guangzhou, China), from 2000 to 2017. The patients were followed up for 3 to 148 months (median follow-up time is 103 months; Supplementary Table S1). Pathologic diagnosis, as well as mitoses and stromal overgrowth status, was confirmed by

two pathologists independently. All samples were collected with informed consents according to the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). Written informed consents were obtained from all patients before recruitment. All procedures performed in this study were in accordance with the ethical standards of the institutional research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Separation and culture of primary cells from breast PTs

Breast PT cells were extracted from the breast PT tissues at the Breast Tumor Center (Supplementary Table S2), Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University as described previously (14). Briefly, the sample tissues were mechanically disaggregated and digested with collagenase type III (1 mg/mL; Worthington) at 37°C with agitation for 1.5 hours in DMEM. The dissociated tissues were filtered through 200 molybdenum, 70- μ m cell strainer, followed by centrifugation at 1,500 rpm for 10 minutes. The cells were collected and seeded in medium with 15% serum and supplements (EGF 20 ng/mL, Peprotech; insulin 10 μ g/mL, Sigma; hydrocortisone 0.5 mg/mL, Sigma).

TAMs were isolated from eight fresh breast malignant PT samples as described previously (15, 16), with slight modifications. Briefly, the tissues were minced into small (1–2 mm) pieces and digested with 5% FBS DMEM containing 2 mg/mL collagenase I and 2 mg/mL hyaluronidase (Sigma) at 37°C for 2 hours. The cells were sequentially filtered through 500- μ m mesh, 100 and 70- μ m cell strainer. The cells were then centrifuged in a Beckman Allegra X-15R centrifuge at 2,500 rpm for 20 minutes with 1-mL cell suspension above 5 mL 45% Percoll (GE Healthcare) in the middle and 5-mL 60% Percoll at the bottom in a 15-mL tube. Mononuclear cells were collected from the cell layer in the interphase between 45% and 60% Percoll.

Macrophage chemotaxis assay

***In vitro* assay.** Monocytes were added to the top chamber and recombinant CCL5 or control media were added to the bottom compartment. In another set of experiments, malignant PT cells or conditioned medium with or without neutralizing antibodies or maraviroc were added to the bottom compartment. After 4 hours at 37°C, the monocytes attached to the lower side of the wells were counted.

***In vivo* assay.** Peripheral blood mononuclear cells from healthy donors were resuspended in PBS containing 1% FBS and incubated with antibody against CD14 microbeads for 30 minutes at 4°C, and then CD14⁺ monocyte cells were resuspended in PBS containing 1% FBS and incubated with CD31 anti-human Alexa Fluor 647 antibody. Then, the CD14⁺ subset of monocytes was separated by magnetic beads. Before the marked cells were injected through tail vein (1×10^6 cells per mouse), the PDX bearing mice were intraperitoneally injected with or without CCR5 inhibitor (10 mg/kg, twice daily for 2 days). After 24 hours of tail vein injection, all PDXs were harvested for live imaging.

Patient-derived xenograft experiments

To establish patient-derived xenografts (PDX), malignant breast PT specimens were collected from patients who underwent tumor resection at Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University between 2017 and 2018. The clinical features of patients were provided in Supplementary Table S6. Detailed

procedure is as described previously (17). Briefly, 6-week-old NOD-SCID female mice were anaesthetized by isoflurane. The malignant PT samples were minced into 1-mm³ sized fragments and imbedded directly into the mammary fat pads to get the first generation of the PDX. Once the PDX of first generation reached diameter of 1 cm, they were harvested and then minced into 1-mm³ sized fragments and imbedded directly into the mammary fat pads to get the second generation of PDX for treatments. The laboratory animal facility has been accredited by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and the Institutional Animal Care and Use Committee (IACUC) of Guangdong Laboratory Animal Monitoring Institute approved all animal protocols used in this study.

Statistical analysis

The *in vitro* data were depicted as mean \pm SD of three independent experiments performed in triplicate. All statistical analyses were performed using SPSS 16.0 statistical software package (SPSS). Student *t* test and one-way ANOVA was used to compare CCL5 expression levels between the PTs with different tumor grades, whereas χ^2 test was used to analyze the relationship between CCL5 expression and clinicopathologic status. Kaplan–Meier curves and log-rank test were used to compare overall survival (OS) and disease-free survival (DFS) in different patient groups. The ROC curve was generated according to the physiologic diagnoses and the CCL5 levels in the sera of the patients to estimate the efficacy of CCL5 as the prognostic marker for PTs using SPSS. In all cases, $P < 0.05$ was considered statistically significant.

Results

CCL5 is the key cytokine that is highly secreted by malignant PT cells

Our previous studies revealed that malignant PTs had higher macrophages density than benign PTs. In addition, the macrophages in malignant PTs exhibited typical TAM phenotype (12). These findings suggested that once macrophages were recruited into tumor tissue, they were induced into TAM phenotype by tumor microenvironment. Therefore, we hypothesized that malignant PTs cells secreted certain cytokines to recruit and repolarize macrophages. The conditional medium (CM) of malignant PTs cells was used to test whether MDMs could be recruited in a Transwell system. We found that CM of malignant PT cells significantly recruited macrophages to penetrate the membrane to the lower chamber, whereas CM of benign PT cells was not able to recruit macrophages (Fig. 1A). Our group as well as other groups previously revealed that CCL17, CCL18, CCL22, and IL10 were highly expressed by TAMs (12, 14). To test whether CM of PT cells could induce TAM polarization, MDMs were treated with the CM of PT cells for 6 days and then cultured in growth medium for 2 days. The secreted cytokines in the culture medium of MDMs were determined. MDMs were treated with the CM of malignant PT cells for 6 days and then continued to be cultured in growth medium of MDMs for 2 days (Fig. 1B). These results suggested that cytokines secreted from malignant PT cells could be involved in recruiting and repolarizing macrophages.

To investigate the key cytokines secreted by malignant PTs that recruit and induce the polarization of TAMs, an antibody array was applied to investigate the cytokines secreted by malignant PT cells. Eighty cytokines were examined in the culture medium of

primary malignant or benign PT cells. Among them, four cytokines were found to be secreted at higher levels by malignant PT cells as compared with benign PT cells, namely CCL2, CCL7, IGFBP1, and HGF. On the other hand, IL10 and osteoprotegerin were significantly lower in culture medium of malignant PT cells than that of benign PT cells (Fig. 1C and D). The results were further verified by ELISA assay using culture medium of eight primary malignant or primary benign PT cells. We found that CCL5 level was much higher in the culture medium of malignant PT cells than that of benign PT cells (Fig. 1E). To elucidate which of these cytokines was the key driver that induced TAM polarization, RNAi was applied to downregulate these cytokines in malignant PT cells prior to coculturing these PT cells with MDMs. Our previous studies revealed that CCL18 was highly expressed by TAMs and was the key cytokine that drove the malignant progression of PTs (12, 14). Here, we found that CCL18 dramatically decreased in the culture medium of MDMs when they were cultured in the CM of CCL5 deficient malignant PT cells (Fig. 1F; Supplementary Fig. S1A), suggesting that CCL5 might be the driving factor that induced TAM polarization in the tumor microenvironment.

CCL5 is highly expressed in malignant PT tissues and indicates poor outcome of patients with PT

To further confirm whether CCL5 correlated with the malignant progression of PTs, we examined the presence of CCL5 in 323 PT samples by IHC, including 199 benign, 46 borderline, and 78 malignant PTs. The increased CCL5 level correlated with malignant progression of PT (Fig. 2A; Supplementary Table S3). CCL5 activity is mainly mediated through its binding to CCR5. CCR5 has been reported to be predominantly expressed on T cells, macrophages, and dendritic cells (18). To examine whether the expression of CCR5 was dominantly expressed on the membrane of macrophages, we performed immunofluorescence (IF) staining in PT tissues and found that the expression of CCR5 was well colocalized with CD163, the marker of M2 macrophages, whereas inducible nitric oxide synthase, the marker of M1 macrophages (19), was seldom expressed in the tissues of PTs (Supplementary Fig. S1B and S1C). In addition, CCR5 and CD163 were minimally expressed in benign PTs, elevated in borderline PTs, and further increased in malignant PTs (Supplementary Fig. S1C). We then examined the expression of CCR5 by IHC in 323 PT samples, and found that CCR5 was highly expressed in the macrophages of malignant PTs with increasing macrophages density from normal breast tissue to benign, borderline, and malignant PTs (Fig. 2A). To quantify the level of CCL5, we measured the CCL5 mRNA levels in fresh frozen PT tissues and primary cells isolated from PTs. Using qRT-PCR, we found that the mRNA levels of CCL5 from fresh frozen tissues and primary cells isolated from PTs were progressively increased with PT malignancy (Supplementary Fig. S1D and S1E). In addition, the secreted CCL5 in the culture medium of eight cases of primary cells isolated from normal breast tissues, benign, borderline, and malignant PT tissues or primary cells PTs was detected by ELISA. We found that malignant PT cells produced highest CCL5 compared with other groups (Supplementary Fig. S1F).

Next, we tested whether CCL5 had clinical prognostic value for patients with PT. We followed the case of 323 patients with PT for 3 to 148 months (median follow-up time is 103 months). We then analyzed the association of CCL5 expression with the clinicopathologic status of PTs (Supplementary Table S3). The

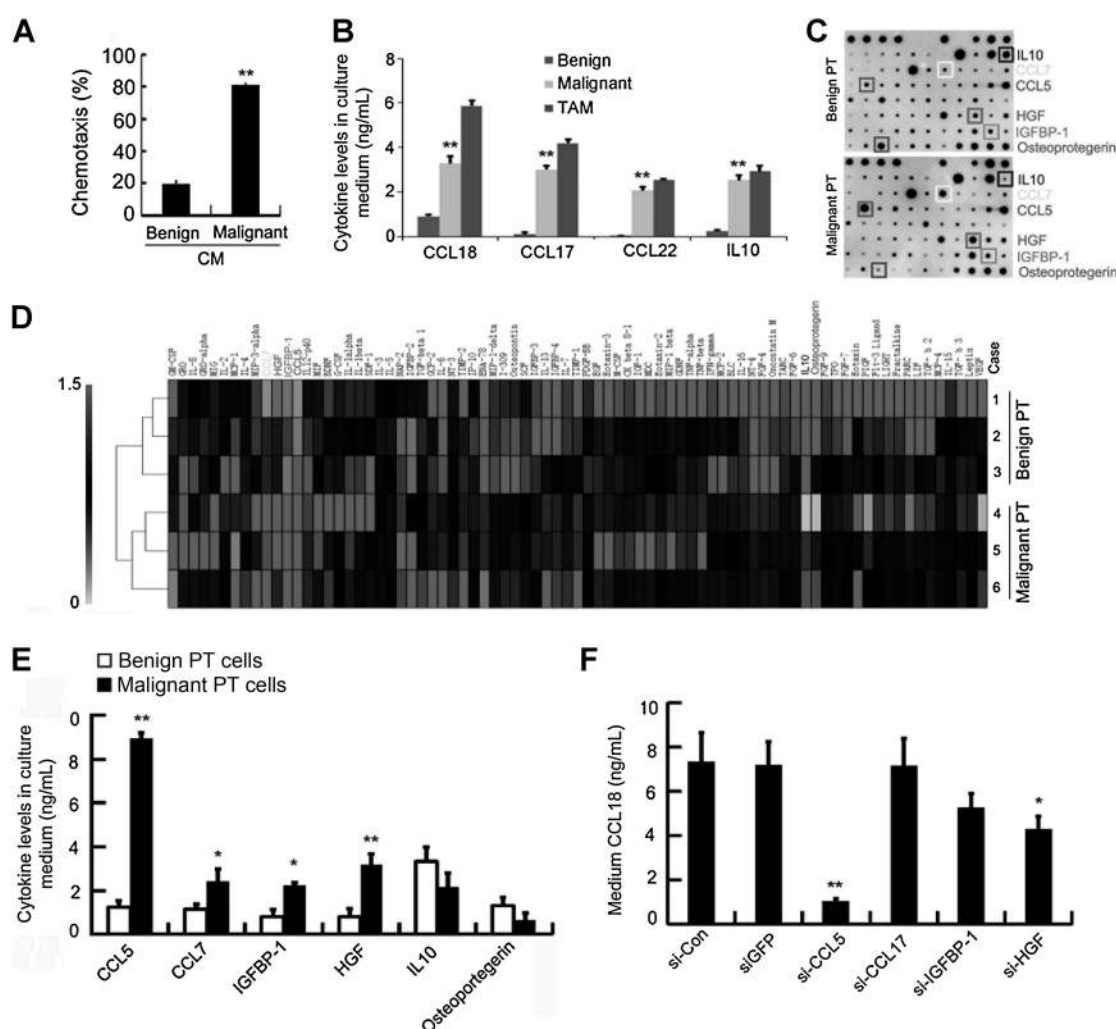
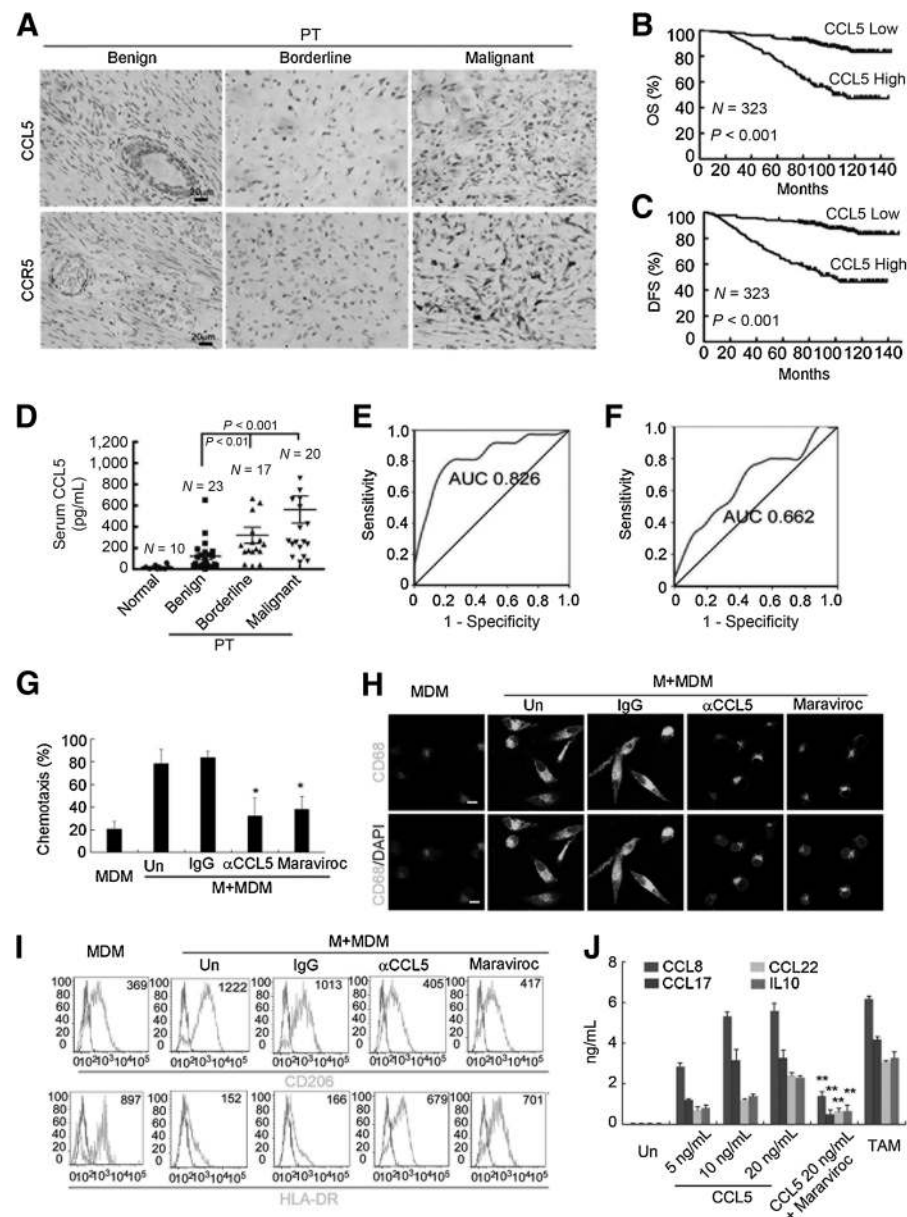


Figure 1.

Malignant PT cells secrete CCL5 and induce TAM repolarization. **A**, The CM of malignant PT cells significantly induces chemotaxis of monocytes. The chemotaxis effect of monocyte in response to the CM of PT cells was determined using a transwell assay. Monocytes were cultured in DMEM supplemented with 10% heat-inactivated human AB serum in the upper chamber. The lower chamber contained the same medium with 30% CM of benign or malignant PT cells (mean \pm SD; $n=5$ independent experiments; **, $P < 0.01$, compared with macrophage treated with CM from benign PT cells. P values were obtained using two-tailed Student t test). **B**, The TAM-related cytokine levels were increased in the cultured media of MDMs treated with the CM of malignant PT cells. The MDMs were treated in CM of benign or malignant PT cells for 6 days and then cultured in growth medium for 2 days. The indicated cytokines in the growth medium were examined by ELISA. The cytokines in the cultured medium of the primary TAMs isolated from malignant human breast PT tissue were used as positive controls (mean \pm SD; $n=5$ independent experiments; **, $P < 0.01$, compared with MDMs treated with CM of benign PT cells. P values were obtained using two-tailed Student t test). **C** and **D**, Cytokines that are predominantly expressed by the malignant PT cells. A total of 1×10^6 benign or malignant PT cells were plated in 60-mm plates with 5 mL DMEM + 15% FBS for 48 hours, and then the supernatants were collected for cytokine array. **C**, A typical view of the cytokine array indicating protein levels in the CM of benign or malignant PT cells. **D**, The heatmap summarizing the relative signal intensity of indicated cytokines in the arrays. Cytokine array reveals that six cytokines (highlighted in colorful font) were differently produced by the malignant or benign PT cells. **E**, Among all cytokines in the cultured media of PT cells from benign or malignant PT cells, CCL5 showed the highest increment among the upregulated cytokines that were produced by malignant PT cells as confirmed by ELISA assay. Bar graphs represent mean \pm SD of 8 samples in each group. **, $P < 0.01$ and *, $P < 0.05$ as compared with benign PT cells. **F**, CCL18 level secreted by MDMs significantly decreased when MDMs were cultured in the CM of malignant PT cells transfected with CCL5 siRNA. Before this experiment, the malignant PT cells were first transfected with indicated siRNA for 48 hours before the collection of the CM of the malignant PT cells for culturing MDMs (refer to **B** for culture method). CCL18 level in the MDM culture medium was determined by ELISA. Bar graphs correspond to mean \pm SD of experimental triplicates from three cases of primary cells. **, $P < 0.01$, and *, $P < 0.05$ as compared with MDMs of control group.

expression of CCL5 increased with higher tumor grade, mitotic activity, and stromal overgrowth ($P < 0.001$), but was not associated with age and the size of tumor (Supplementary Table S3). The expression of CCL5 was also more abundant in the PTs with local recurrence and distal metastasis ($P < 0.001$; Supplementary Table S3). Furthermore, Kaplan–Meier survival curve demonstrat-

ed that patients with low CCL5 expression (staining index, SI ≤ 4 ; refer to the Supplementary Materials and Methods and refs. 12, 15) have a longer OS and DFS than those with high CCL5 expression ($P < 0.001$; Fig. 2B and C). ELISA detection of the serum levels of CCL5 from the patients with PT showed that patients with malignant PTs had significantly higher serum levels

**Figure 2.**

The PT cell-secreted CCL5 is associated with malignant progression of PTs and can induce macrophages to a TAM-like phenotype. **A**, IHC staining indicated elevated CCL5 and its corresponding receptor CCR5 in the malignant breast PT tissues compared with benign or borderline breast PT tissues. Scale bar, 20 μm. Kaplan-Meier OS curve (**B**) and DFS curve (**C**) of patients with breast PT with low and high CCL5 within a median follow-up period of 148 months. The median OS and the median DFS are 112 months and 98 months in CCL5-high group, respectively. The CCL5-low group has not reached the median OS and the median DFS time. **D**, Serum CCL5 level increases in the patients with breast PTs, as determined by ELISA. Bar graphs correspond to mean \pm SD. **E** and **F**, ROC curve shows the value of serum CCL5 for distinguishing aggressive from benign PTs. Benign versus borderline and malignant PTs (**E**). Borderline versus malignant PTs (**F**). **G**, Blocking CCL5-CCR5 axis prevents the malignant PT cell-induced monocyte chemotaxis. Monocytes were cultured in the upper chamber, whereas malignant PT cells (indicated as "M") cultured in the lower chamber of transwell with anti-CCL5-neutralizing antibody or maraviroc in the culture medium. Anti-CCL5-neutralizing antibody or maraviroc significantly reduced the migration of MDMs from the upper to the lower chamber. (Bars correspond to mean \pm SD of three independent experiments with MDMs from 3 healthy donors and primary malignant PT cells from 3 individual patients. *, $P < 0.05$ compared with control). **H** and **I**, MDM polarization induced by malignant PT cells could be reversed by blocking CCL5-CCR5 axis. MDMs were replated alone or cocultured with malignant PT cells in transwell as described in **G** in the presence of control IgG, anti-CCL5-neutralizing antibody, or CCR5 inhibitor for 6 days. **H**, Fluorescent CD68/DAPI staining in macrophages. Scale bar, 20 μm. **I**, Expression of CD206/HLA-DR in macrophages. The histograms are representatives of five independent experiments of MDMs from 5 healthy donors and primary malignant PT cells from 5 individual patients. Numerical values denote the mean fluorescence intensity (MFI). **J**, Cytokine levels in the culture media of macrophages cocultured with malignant PT cells as in **A** (mean \pm SD, $n = 5$ independent experiments with MDMs from 5 healthy donors and TAMs isolated from 5 individual patients; **, $P < 0.01$ compared with control). Maraviroc treatment in the lower chamber could decrease the medium level of CCL18, CCL17, CCL22, and IL10 to almost background levels in the MDM and malignant PT cell coculture model. (Also see Supplementary Fig. S2).

of CCL5 than those with benign and borderline PTs (Fig. 2D; Supplementary Table S4). We, thus, used the ROC curve to evaluate the efficacy of serum CCL5 in patients with PT as a diagnostic marker. It showed that CCL5 serum level could distinguish aggressive cases from benign PTs (Fig. 2E and F). In addition, multivariate Cox regression analyses demonstrated that CCL5 ($P < 0.001$), stromal overgrowth ($P < 0.001$), and tumor grading ($P < 0.001$) were independent prognostic predictors for local recurrence-free survival (LRFS; Supplementary Table S5). These results suggest that the CCL5 level is associated with malignant progression of PT, and the levels of CCL5 can be used to predict the outcome of LRFS with PT.

Malignant PT recruits and induces macrophages to a TAM-like phenotype via CCL5

High density of TAMs has been linked with cancer progression, including breast PTs (12, 15), suggesting that macrophages are recruited into tumor tissues and then undergo repolarization into TAM phenotype. The CCL5-CCR5 axis has been reported to recruit macrophages in several pathologic processes, such as obesity and breast cancers (20, 21). We confirmed the ability of CCL5 to recruit monocytes by using monocytes from healthy donors (Supplementary Fig. S2A). In addition, we tested the effects of blocking CCL5-CCR5 using anti-CCL5-neutralizing antibody or maraviroc, an approved CCR5 inhibitor, which was originally developed for HIV patients as viral entry-blocking inhibitor (22). When monocytes were cocultured with malignant PT cells in the Transwell, anti-CCL5-neutralizing antibody or maraviroc could significantly reduce the migration of monocytes through the membrane to almost the background level (Fig. 2G). These observations suggested that the CCL5-CCR5 axis played a key role for malignant PT cells to recruit macrophages.

We then tested whether CCL5-CCR5 axis could induce the repolarization of macrophages. Although TAMs are heterogeneous, the TAMs predominantly showed a M2-like polarization with CD206^{high}/HLA-DR^{low} phenotype (16, 23). Macrophages treated with CCL5 became stretched and elongated and exhibited a CD206^{high}/HLA-DR^{low} phenotype (Supplementary Fig. S2B and S2C) similar to primary TAMs, whereas CCR5 inhibition blocked the phenotype change of macrophages induced by CCL5 (Supplementary Fig. S2B and S2C). Consistently, blocking the CCL5-CCR5 axis with anti-CCL5-neutralizing antibody or maraviroc dramatically reversed the stretched morphology (Fig. 2H) and CD206^{high}/HLA-DR^{low} phenotype of macrophages induced by coculturing with malignant PT cells (Fig. 2I). TAMs produced high amounts of the protumor cytokines, CCL18, CCL17, CCL22, and IL10 (15, 16, 24). In accordance with the changes in morphology and surface markers, MDMs treated with increasing doses of CCL5 produced higher levels of these cytokines than untreated macrophages (Fig. 2J), indicating functional activation of TAMs. Again, maraviroc could decrease the CCL18, CCL17, CCL22, and IL10 levels to almost background levels in the medium (Fig. 2J). Together, these data suggest that CCL5-CCR5 axis is essential for malignant PT cells to recruit macrophages and induce the polarization of TAMs. We also knocked down other known CCL5 receptors (CCR1 and CCR3) by RNAi. We found when monocytes or MDMs were treated with CCL5, CCR1, or CCR3, the knockdown did not prevent the CCL5-induced recruitment of monocytes or the polarization toward TAMs (data not shown).

The repolarized TAMs enhanced and maintained the malignant phenotype of PT cells via the positive feedback loop constitutes of CCL5-CCR5 and CCL18-PIPTNM3 axis

We have exhibited that malignant PT cells triggered the repolarization of TAMs via the CCL5-CCR5 axis. In our previous studies, we showed that TAMs secreted CCL18 to promote the malignant progression of PTs via binding to PIPTNM3 (12). Therefore, we hypothesized that once the macrophages were repolarized by malignant PT cells, they would in turn enhance the malignancy of PT cells. The CCL18 were examined by ELISA in the MDM-malignant myofibroblast coculture system. CCL18 gradually increased within 12 hours of coculture, rapidly increased by the second day, and steadily increased even after 4 days of coculture. When the CCL5-neutralizing antibody or the CCR5 inhibitor was introduced into the coculture system, the CCL18 only increased minimally and maintained at much lower level than the untreated groups (Fig. 3A). As for CCL5 level, it was only slightly increased in the first 2 days, then dramatically increased on the third day and keep increasing steadily in the following days (Fig. 3B). When adding anti-CCL18 antibody in the coculture system, CCL5 level decreased from the third day onwards (Fig. 3B). These observations implied that the communication via CCL5-CCR5 axis and CCL18-PIPTNM3 axis between malignant PT cells and MDMs would constitute a positive feedback loop to induce the repolarization of TAMs, and further maintain and enhance the malignancy of PTs.

Therefore, the malignant phenotypes of PT cells were evaluated in the coculture system. Myofibroblasts are the major malignant component of PTs, and are hallmarked by the highly expressed α -SMA, fibroblast activation protein (FAP), and high invasion ability (14, 25, 26). The freshly isolated primary myofibroblasts from malignant PTs were cultured in growth medium overnight or maintained in the growth medium for 6 days, or parallelly cocultured with MDMs for 6 days. Then, the α -SMA expression was evaluated by IF staining. The myofibroblasts single-cultured overnight or in 6 days of coculture with MDMs showed similar α -SMA levels, which were much higher than that of myofibroblasts only maintained in the growth medium for 6 days (Fig. 3C). When CCL5-CCR5 axis were blocked by CCL5-neutralizing antibody or the CCR5 inhibitor, the levels of α -SMA declined and did not maintain as high as in the coculture model. Consistently, blocking CCL18-PIPTNM3 axis with CCL18 antibody also decreased the α -SMA levels of myofibroblasts in the coculture model (Fig. 3C). In addition, the expression of FAP showed similar trend in each of the treatment groups (Fig. 3C).

The migration and invasion ability of the primary myofibroblasts isolated from malignant PTs were evaluated by Transwell assays. The primary myofibroblasts isolated from malignant PT were treated as described above. The migration and invasion ability of myofibroblasts declined when they were single-cultured in growth medium for 6 days as compared with freshly isolated myofibroblasts single-cultured overnight. Myofibroblasts cocultured with MDM for 6 days maintained the migration and invasion ability of myofibroblasts as compared with the overnight single-cultured myofibroblasts (Fig. 3D and E). On the other hand, the neutralizing antibodies of CCL5 or CCL18 and the CCR5 inhibitor treatment decreased migration and invasion ability of myofibroblasts in coculture model. Myofibroblasts are known to have an increased ability to induce collagen gel contraction, representing the ability of reorganization of the extracellular matrix (27, 28). Therefore, collagen contraction assay was

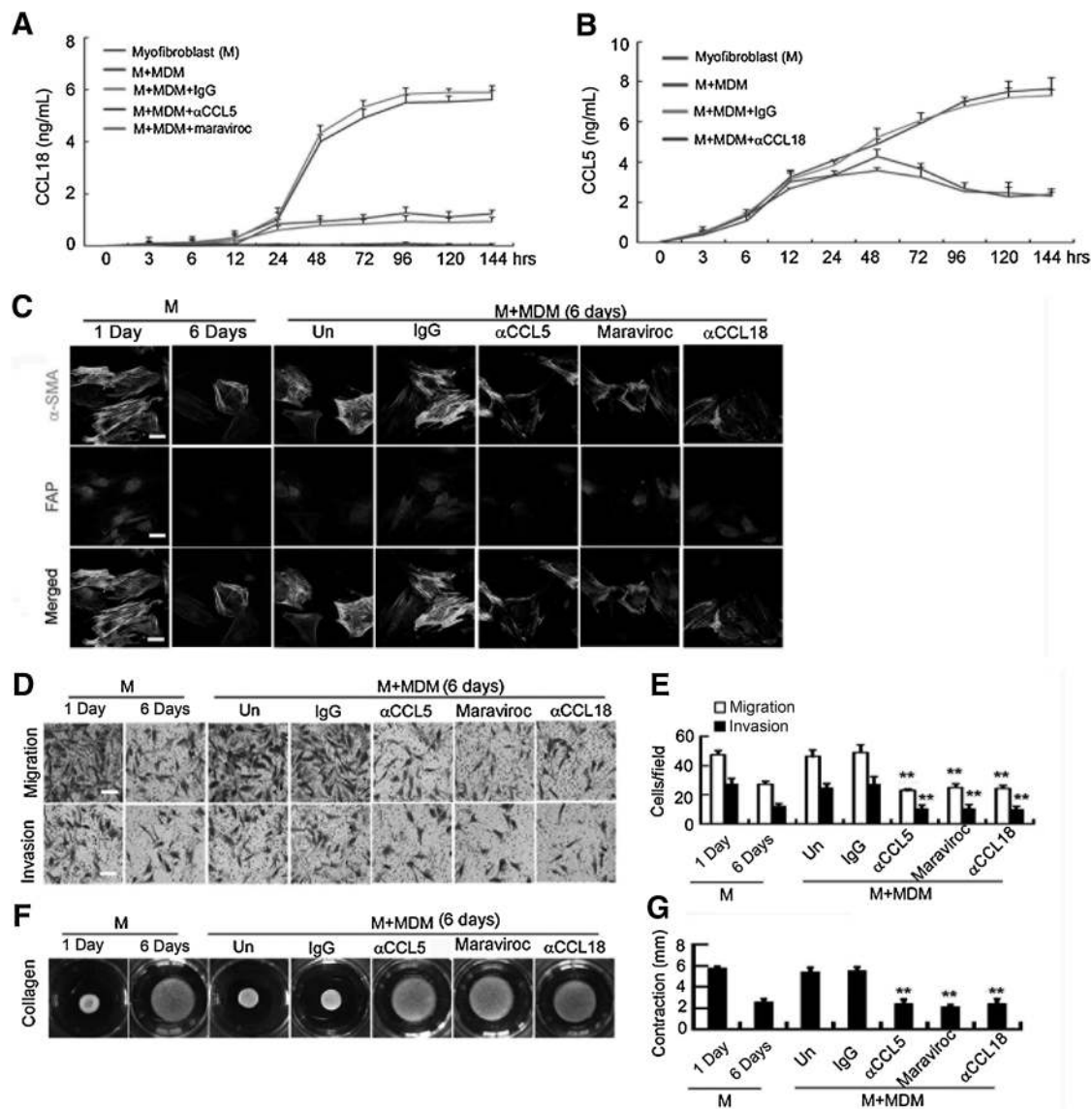


Figure 3.

The positive feedback loop of CCL5-CCR5 and CCL18-PIPTNM3 between myofibroblasts and TAMs enhances and maintains the malignant phenotypes of PT cells.

A, MDMs cocultured with malignant PT (denoted as "M") cells induce the expression of CCL18. MDMs (lower chamber) were replated alone or cocultured with malignant PT cells (upper chamber) in transwell apparatus with or without control IgG, anti-CCL5-neutralizing antibody, or CCR5 inhibitor for 6 days. CCL18 levels in the culture media of MDMs were measured by ELISA at indicated days. All values are mean \pm SD, $n = 5$ independent experiments of MDMs from 5 healthy donors and primary malignant PT cells from 5 individual patients. **B**, Malignant PT cells cocultured with MDMs promote and maintain the CCL5 expression of malignant PT cells. Malignant PT cells (lower chamber) were replated alone or cocultured with MDMs (upper chamber) in transwell apparatus with or without control IgG, anti-CCL18-neutralizing antibody for 6 days. CCL5 levels in the culture media of malignant PT cells were measured by ELISA at indicated days. All values are mean \pm SD, $n = 5$ independent experiments of MDMs from 5 healthy donors and primary malignant PT cells from 5 individual patients. **C**, Coculturing with MDMs maintains the α -SMA and FAP expression of malignant PT cells. IF analysis of α -SMA and FAP in malignant PT cells alone or in the coculture system as described in **B**. MDMs from 5 healthy donors and primary malignant PT cells from 5 individual patients were used. The representative images were used for this figure. Scale bar, 20 μ m. **D** and **E**, Coculturing with MDMs maintains the invasive phenotype of malignant PT cells. The malignant PT cells were single-cultured or cocultured with MDMs as described in **B** and then harvested for migration or invasion assay in the transwell. **D**, The representative images of migrated and invaded PT cells in the transwell. **E**, The statistical analysis of migration and invasion assay of malignant PT cells. Bars correspond to means \pm SD of MDMs from 8 healthy donors and primary malignant PT cells from 8 individual patients with experimental triplicates for each sample. **, $P < 0.01$. **F** and **G**, Coculturing with MDMs maintains the gel contraction ability of malignant PT cells. The malignant PT cells were single-cultured or cocultured with MDMs as described in **B** and then harvested for gel contraction assay. **F**, Representative images of collagen gel contraction for PT cells. **G**, The statistical analysis of gel contraction assay of malignant PT cells. Bars correspond to means \pm SD of MDMs from 8 healthy donors and primary malignant PT cells from 8 individual patients with experimental triplicates for each sample. **, $P < 0.01$.

used to test the primary myofibroblasts isolated from malignant PTs. Similar to the observation above, we observed that freshly isolated myofibroblasts showed much stronger ability of contracting collagen gels than the myofibroblasts single-cultured for 6 days in growth medium. When cocultured with MDMs for 6 days, myofibroblasts maintained the ability of contracting collagen gels as strongly as the freshly isolated myofibroblasts (Fig. 3F and G). Again, if the neutralizing antibodies of CCL5 or CCL18 and the CCR5 inhibitor were introduced into the coculture system, the ability of the myofibroblast to contract collagen gels decreased dramatically (Fig. 3F and G). Together, these findings suggested that the repolarized TAMs maintained the malignant phenotype of PT cells via the positive feedback loop of CCL5-CCR5 and CCL18-PIPTNM3.

Furthermore, macrophages are frequently found in close proximity with collagen-producing myofibroblasts (4), and there is strong evidence that this interaction of macrophages and myofibroblasts are reciprocal (29). Macrophages produce profibrotic mediators that directly activate fibroblasts, including TGF- β 1 and PDGF (3). Macrophages also produce insulin-like growth factor-I, which stimulates the proliferation and survival of fibroblasts and promotes collagen synthesis (5). In malignant PTs, there are still a lot of quiescent or normal mesenchymal fibroblasts mixed with the myofibroblasts (Supplementary Fig. S3A). Therefore, we further cocultured TAMs isolated from malignant PTs with fibroblasts isolated from benign PT tissues to evaluate whether the interaction of TAMs with fibroblasts induced the myofibroblast differentiation, thus enhancing the malignant progression of PTs. Compared with single-cultured fibroblasts from benign PT, fibroblasts cocultured with primary TAMs had a significant higher level of α -SMA and FAP (Supplementary Fig. S3B) and higher ability of migration, invasion, and collagen contraction (Supplementary Fig. S3C–S3F), suggesting myofibroblast differentiation of the fibroblasts. Again, the myofibroblast differentiation could be reversed by the treatment of neutralizing antibodies of CCL5 or CCL18 and the CCR5 inhibitor (Supplementary Fig. S3A–S3F). These data suggest that once the macrophages are repolarized to TAMs, they induce the myofibroblast differentiation of mesenchymal fibroblasts, thus, further promoting the malignancy of malignant PTs.

The CCL5-CCR5 axis activates AKT signaling to repolarize the TAMs

CCL5-CCR5 contributes to the activation of AKT, MAPK/ERK, JNK-STAT, and NF- κ B pathways (30). To identify which of these pathways mediated the polarization of TAM, MDMs, and primary TAMs isolated from malignant PTs were collected for Western blotting. We found that p-AKT level significantly increased and p-I κ B slightly increased, whereas p-STAT3 level decreased in the TAMs, as compared with MDMs (Fig. 4A). Then, we further treated MDMs with increased amount of CCL5 with or without CCR5 inhibitor. Among these pathways, only p-AKT was increased in a dose-dependent manner, and was decreased with CCR5 inhibitor treatment (Fig. 4B). On the other hand, p-STAT3 was decreased upon CCL5 treatment and reversed by CCR5 inhibitor (Fig. 4B). Furthermore, when MDMs were cocultured with myofibroblasts isolated from malignant PTs, the CCL5-neutralizing antibody or CCR5 inhibitor decreased the p-AKT level of macrophages in the coculture model, while the p-STAT3 levels increased (Fig. 4C).

Activation of AKT pathway has been linked to polarization of M2 macrophages (31, 32). Therefore, we examined whether the

abrogation of AKT activation would suppress the CCL5-induced TAM polarization. In the coculture model, the AKT inhibitor as well as the CCL5-neutralizing antibody or CCR5 inhibitor decreased the malignant PT cell-induced monocyte recruitment (Fig. 4D). Furthermore, these treatments also dramatically reversed the stretched morphology (Fig. 4E) and CD206^{high}/HLA-DR^{low} phenotype (Fig. 4F) of macrophages induced by malignant PT cells in the coculture model. These results suggested that CCL5-CCR5 axis induced the polarization of TAMs via activating AKT pathways.

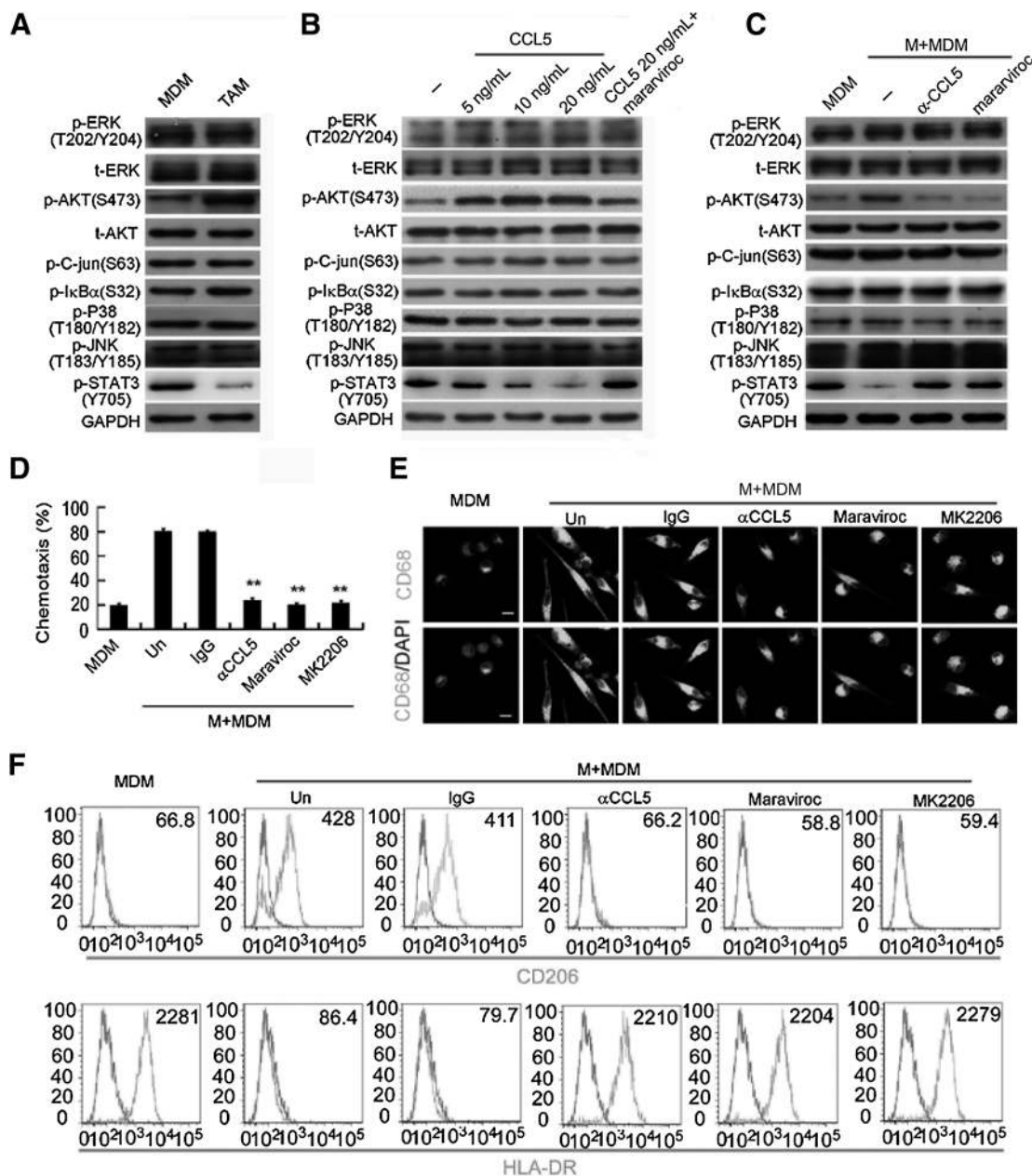
Blocking the loop of CCL5-CCR5 axis and CCL18-PIPTNM3 axis prevents breast PT growth and metastasis

Our previous studies showed that TAMs secreted CCL18 to induce myofibroblast differentiation, accelerate tumor growth, and promote metastasis of breast PT xenografts (12). To investigate the role of the positive feedback loop of CCL5-CCR5 axis and CCL18-PIPTNM3 axis on PT formation and progression *in vivo*, we inoculated the primary benign or malignant PT cells with or without MDMs into the mammary fat pads of athymic nude mice ($n = 8$ per group), and evaluated tumor growth and metastasis. When the xenografts became palpable, CCL5- or CCL18-specific neutralizing antibody (1 mg/kg twice weekly) or isotype IgG (1 mg/kg twice weekly) were injected via the tail vein twice weekly. Maraviroc, the CCR5 inhibitor, was also used to estimate the potential therapeutic effect (10 mg/kg, i.p., daily). We found that compared with malignant PT cells inoculated alone, additional injection of MDMs significantly increased the tumor formation efficiency ($P < 0.01$; Fig. 5A) and accelerated the tumor growth of xenografts ($P < 0.01$; Fig. 5B). CCL5- or CCL18-specific neutralizing antibody, as well as maraviroc, markedly inhibited the tumor formation and lowered the tumor growth of xenografts in the malignant PT cells plus MDMs inoculation groups ($P < 0.01$, Fig. 5A and B). More importantly, if the feedback loop of CCL5-CCR5 axis and CCL18-PIPTNM3 axis were double-blocked by the combination of maraviroc and CCL18-specific neutralizing antibody, the tumor growth and metastasis were further inhibited (Fig. 5A and B).

To further evaluate whether the loop of CCL5-CCR5 axis and CCL18-PIPTNM3 axis regulated myofibroblast differentiation and macrophage polarization *in vivo*, we examined the protein levels of α -SMA, CD163, CCL5, and CCL18 in the xenografts using dual-IF staining. Similar to the results obtained *in vitro*, treatment with CCL5- or CCL18-specific neutralizing antibody or maraviroc significantly decreased the levels of α -SMA CD163, CCL5, and CCL18 in the xenografts (Fig. 5C). As increased migration and invasion of tumor cells are linked with metastasis, we evaluated whether blocking the positive feedback loop of CCL5-CCR5 axis and CCL18-PIPTNM3 axis resulted in decreased metastasis of breast PT tumors. Consistent with the findings *in vitro*, treatment with CCL5- or CCL18-specific neutralizing antibody or maraviroc significantly decreased the number of metastatic nodules and human hypoxanthine-guanine-phosphoribosyltransferase (HPRT) mRNA in the lungs and livers (Fig. 5D; Supplementary Fig. S4A, S4B, S4D, and S4E) and reduce the wet weight of lungs (Supplementary Fig. S4C).

The CCR5 inhibitor maraviroc inhibits PT growth in the PDX model

We have shown that blocking CCL5-CCR5 axis could prevent the macrophage chemotaxis recruited by the malignant PT cells.

**Figure 4.**

CCL5-CCR5 axis repolarizes TAM through activation of AKT signaling. **A**, Active AKT was increased whereas phospho-STAT3 was decreased in TAMs isolated from the malignant PTs. The MDMs were generated from the monocytes of the same patients. Experiments were repeated three times with MDMs from 3 healthy donors, TAMs and primary malignant PT cells from 3 individual patients. **B**, CCL5 treatment activates AKT in a dose-dependent manner in MDMs. MDMs were treated with indicated dose of CCL5 with or without maraviroc. The AKT phosphorylation increased, although STAT3 phosphorylation decreased when CCL5 was present in the culture medium, whereas CCR5 inhibition with maraviroc reversed these effects. Experiments were repeated three times with MDMs from 3 healthy donors, TAMs and primary malignant PT cells from 3 individual patients. **C**, Treatment with the CM of malignant PT cells increased the AKT phosphorylation of MDMs via CCL5-CCR5 axis. The MDMs were treated with CM of malignant PT cells with or without maraviroc. AKT phosphorylation increased, although STAT3 phosphorylation decreased when CM of malignant PT cells was present in the culture medium, whereas CCR5 inhibition with maraviroc reversed these effects. Experiments were repeated three times with MDMs from 3 healthy donors, TAMs and primary malignant PT cells from 3 individual patients. **D**, Blocking AKT activation prevents the malignant PT cell-induced monocyte chemotaxis. Monocytes were cultured in the upper chamber, whereas malignant PT cells cultured in the lower chamber of transwell with anti-CCL5-neutralizing antibody, maraviroc, or AKT inhibitor (MK2206). Blocking AKT activation significantly reduced the migration of MDMs from the upper to lower chamber as well as blocking CCL5-CCR5 axis by anti-CCL5-neutralizing antibody or maraviroc (bars correspond to means \pm SD of three independent experiments with MDMs from 3 healthy donors and primary malignant PT cells from 3 individual patients; **, $P < 0.01$ compared with control). MDM polarization induced by malignant PT cell is reversed by blocking AKT activation. MDMs were replated alone or cocultured with malignant PT cells in transwell apparatus with or without control IgG, anti-CCL5-neutralizing antibody, CCR5 inhibitor, or AKT inhibitor for 6 days. **E**, Fluorescent CD68/DAPI staining in macrophages. Scale bar, 20 μ m. **F**, Expression of CD206/HLA-DR in macrophages was detected by flow cytometry. The histograms are representatives of five independent experiments of MDMs from 5 healthy donors and primary malignant PT cells from 5 individual patients. Numerical values denote MFI.

Next, we used the PDX model of malignant PTs to test whether CCR5 inhibition can prevent the recruitment of macrophage into malignant PTs *in vivo*. The human Alexa Fluor 647-labeled monocytes were injected through tail vein into the PDX-bearing mice (1×10^6 cells per mouse) treated with or without maraviroc. By fluorescent scanning, we found that the tumors from the untreated mice exhibited significantly intensive fluorescence than the treated group (Fig. 6A; Supplementary Fig. S5A). In addition, CD68 staining showed that there were much more human TAMs in the untreated tumors than the maraviroc-treated tumors (Fig. 6B; Supplementary Fig. S5B). These observations suggested that inhibiting CCR5 by maraviroc could significantly reduce the macrophage recruitment by malignant PTs.

Although there is no exact counterpart for CCL18 in rodents, several studies have applied CCR5 inhibitors for cancer treatment in animal models, such as breast and liver cancers (33–35), suggesting an alternative loop works similarly as CCL18–PITPNM3 axis to transit the signal from TAMs to malignant PT cells to promote the aggressive manner of PT cells in human. Therefore, we tested whether maraviroc could treat the malignant PTs in the PDX model. We first examined the existence of mouse-derived macrophages in the PDX and found high density of mouse macrophages in the PDX of malignant PTs (Supplementary Fig. S5C and S5D). To examine whether macrophages from NOD-SCID mice could be polarized and function as MDMs from human healthy donors, we prepared peritoneal macrophages from NOD-SCID mice. Then, the mouse macrophages were cocultured with myofibroblasts from malignant PTs. We found that mouse macrophages could be recruited by the myofibroblasts (Supplementary Fig. S6A). In addition, when mouse macrophages were cocultured with malignant PT cells in the Transwell, anti-CCL5–neutralizing antibody or maraviroc could significantly reduce the migration of the macrophages through the membrane to almost the background level (Supplementary Fig. S6A). We then tested whether myofibroblasts could induce the repolarization of macrophages. Macrophages cocultured with myofibroblasts became stretched and elongated (Supplementary Fig. S6B). TAMs are marked as Arginase 1 highly expressed cells (36). We found that the mouse macrophages cocultured with myofibroblasts exhibited an Arginase 1^{high}/HLA-DR^{low} phenotype (Supplementary Fig. S6C), similar to primary TAMs, whereas CCL5–CCR5 inhibition with anti-CCL5–neutralizing antibody or maraviroc blocked the phenotype change of macrophages induced by myofibroblasts (Supplementary Fig. S6C). Consistently, macrophages in PDX exhibited intensive Arginase 1 staining (Supplementary Fig. S6D). On the other hand, using the *in vitro* coculture model, we examined whether mouse macrophage could enhance and maintain the malignant phenotype of PT cells and found that myofibroblasts single-cultured overnight or in 6 days of coculture with mouse macrophages showed similar α -SMA levels, which were much higher than that of myofibroblasts only maintained in the growth medium for 6 days (Supplementary Fig. S6E). When CCL5–CCR5 axis was blocked by CCL5–neutralizing antibody or the CCR5 inhibitor, the levels of α -SMA declined and were not maintained as high as in the coculture model. The expression of FAP also showed similar trend in each of the treatment groups (Supplementary Fig. S6E). Moreover, myofibroblasts showed decreased migration and invasion ability after being single-cultured in growth medium for 6 days, as compared with freshly isolated myofibroblasts single-cultured overnight. Myofibroblasts cocultured with mouse macrophages for 6 days maintained

migration and invasion ability as compared with the overnight single-cultured myofibroblasts (Supplementary Fig. S6F and S6G). Similarly, the mouse macrophages could maintain the gel contraction ability of myofibroblasts in coculture model and blocking the CCL5–CCR5 axis reversed the gel contraction ability induced by mouse macrophages (Supplementary Fig. S6H and S6I). Therefore, we hypothesized that mouse macrophages were also involved in promoting tumorigenesis of malignant PTs in the PDX model. Consistent with the xenograft experiments, the maraviroc treatment dramatically decreased the growth of malignant PT PDX (Fig. 6C–E; Supplementary Table S6), whereas the doxorubicin treatment only showed moderate therapeutic effect (Fig. 6C–E). Detection of mouse macrophages in the tumors by IHC indicated that maraviroc treatment could significantly decrease the macrophages density (Fig. 6F; Supplementary Fig. S6J) and the doxorubicin treatment did not decrease the macrophages density in the PDX (Fig. 6F; Supplementary Fig. S6J). The Ki67 staining also showed the decreased proliferative levels of malignant PT cells in the PDX treated by maraviroc (Fig. 6F; Supplementary Fig. S6K).

Discussion

In PTs, myofibroblasts are derived from stromal fibroblasts and constitute a major malignant component of tumor mass. Transition of the mesenchymal FMT, indicated by the intensive expression of α -SMA and FAP, has been suggested as a key process in the tumorigenesis of PTs (14). Continuous stimulation by toxic, infectious, metabolic agents, or chronic inflammation leads to continuous myofibroblast differentiation from mesenchymal fibroblasts (37, 38). A case report based on whole-genome sequencing revealed N-RAS mutation with concomitant activation of PI3K/Akt/mTOR in PT (39). In consistent with the observations, our previous studies have shown that CCL18/NF- κ B/*miR-21* axis between TAMs and PT cells activates AKT in malignant PT cells and drives the tumorigenesis of PTs (14). However, the tumorigenesis mechanism of breast PT remains largely unknown.

The interactions of tumor microenvironment and tumor cells have been shown to drive the malignant progression of various cancers. TAMs have been linked with cancer progression in cancers (3, 9–11). Our previous results showed that TAMs were the most abundant stromal cells in tumor microenvironment of PTs and the increased density of TAMs linked to the malignant progression of PTs (12, 14). However, the driving mechanisms of how macrophages were recruited and repolarized in PT microenvironment remain unclear. In our studies, CCL5 was revealed as the key cytokine that was overexpressed and secreted by myofibroblasts of malignant PTs. Furthermore, high CCL5 level serves as an independent prognostic factor of PTs indicating the poor outcome and more aggressive stage of PTs. In our previous studies, we showed that TAMs induced myofibroblasts differentiation and promoted the malignant progression of breast PTs via secreting CCL18, thus activated myofibroblasts through binding the CCL18 receptor, PIPTNM3 (12). Here, we went further by showing that the positive feedback loop of CCL5–CCR5 axis and CCL18–PIPTNM3 axis between myofibroblasts and TAMs of PTs are the key regulators of TAM polarization, which maintained and enhanced the FMT of myofibroblasts. Blocking the positive feedback loop by anti-CCL5 or anti-CCL18–neutralizing antibody dramatically

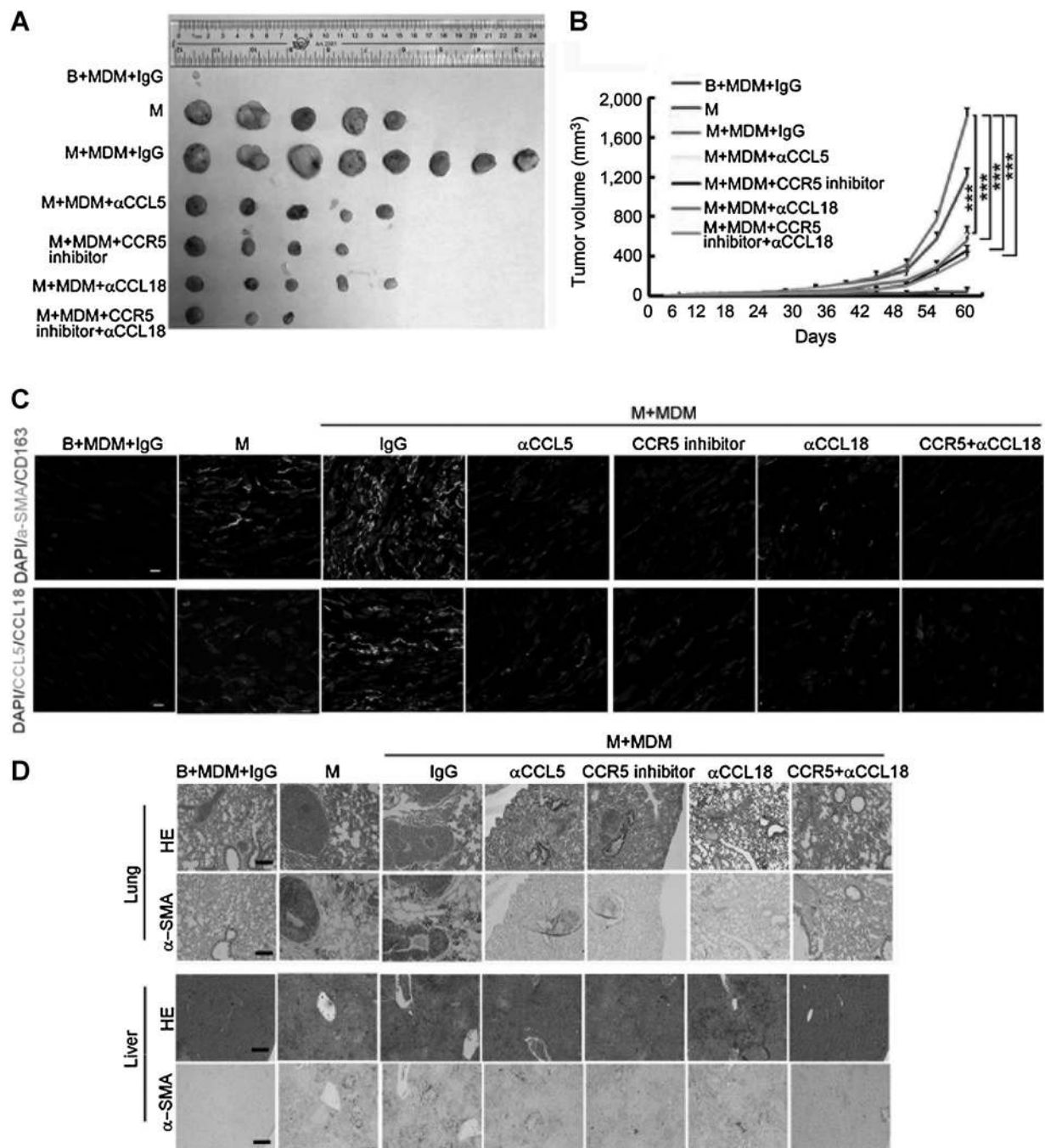


Figure 5.

The loop of CCL5-CCR5 and CCL18-PIPTNM3 accelerates tumor growth, and promotes metastasis of breast PT xenografts. **A**, Inhibiting the CCL5-CCL18 loop suppresses tumor growth. Benign or malignant PT cells and MDM were injected into the mammary fat pads of NOD/SCID mice. Mice were injected with control IgG (1 mg/kg twice weekly), anti-CCL5-neutralizing antibody (CCL5-Ab, 1 mg/kg twice weekly), or anti-CCL18-neutralizing antibody (CCL18-Ab, 1 mg/kg twice weekly) via tail vein injection, CCR5 inhibitor maraviroc (10 mg/kg i.p daily) after the xenografts became palpable ($n = 8$ per group). The neutralizing antibody and inhibitor can both prevent the aggressive growth manner of the malignant PT cells and MDM coculture system. **B**, Tumor growth during the course of each indicated treatment. Error bars show \pm SD ($n = 8$ per group). ***, $P < 0.001$ versus malignant PT cells and MDM inoculated with control IgG-injected group. **C**, Representative images of IF staining of α -SMA and CD163 or CCL5 and CCL18 in the sections from indicated tumor xenografts. Scale bar, 40 μ m. **D**, Representative HE and human α -SMA immunostaining of liver and lung sections from indicated tumor xenografts. Scale bar, 100 μ m.

reverses the myofibroblast differentiation *in vitro* and *in vivo* and, thus, inhibits malignant PT growth *in vivo*. The interaction of PT cells with TAMs plays an essential role in the progression

of malignant PT development, and the positive feedback loop of CCL5-CCR5 axis and CCL18-PIPTNM3 axis represents the key communication of PT cells with TAMs, which drives the

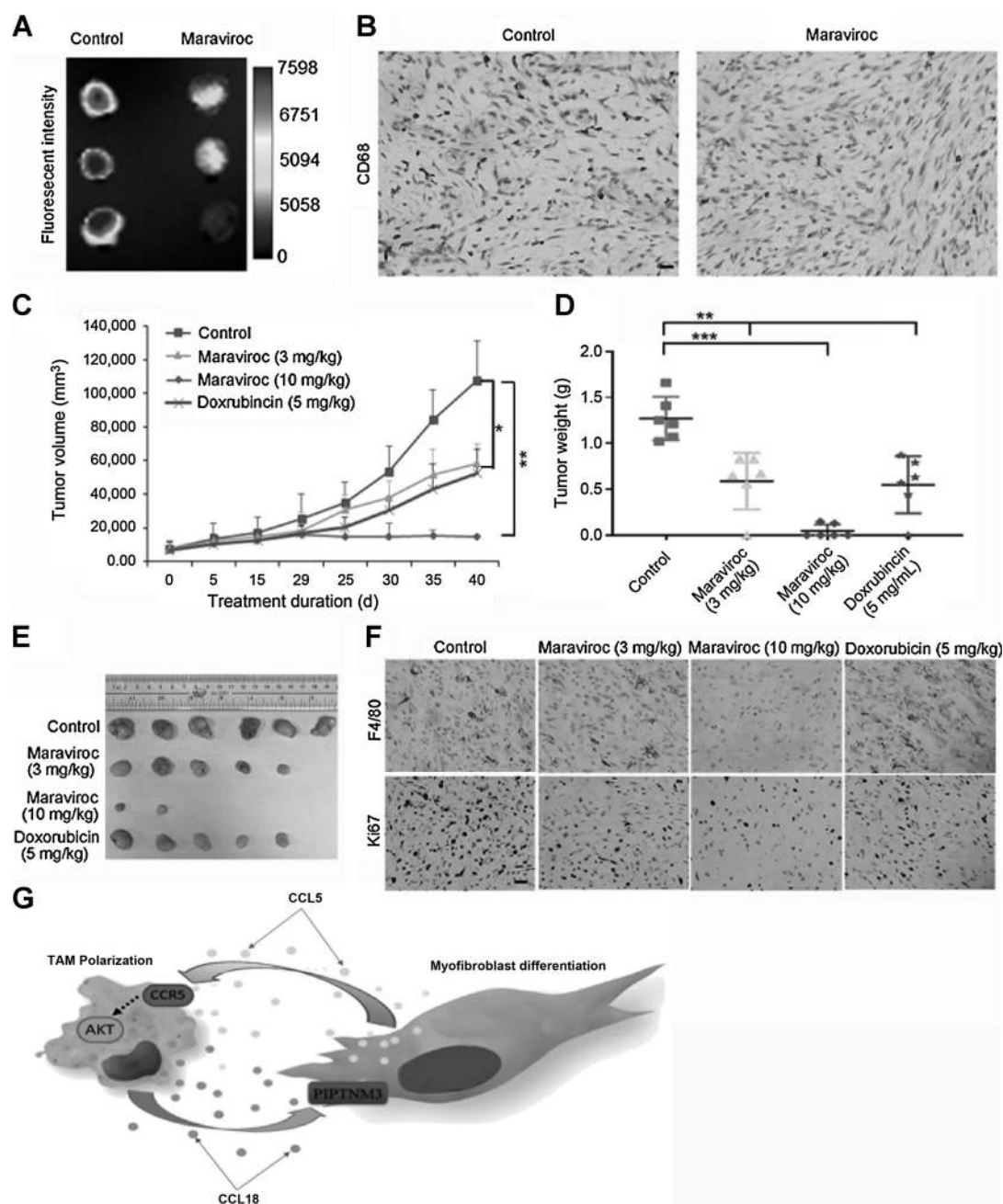


Figure 6.

Treatment of maraviroc suppresses the PDX growth of malignant PTs. **A** and **B**, The CCR5 inhibitor maraviroc prevents the recruitment of macrophages into the tumor tissue by malignant PT. The monocytes from healthy human donors were isolated with CD14 magnetic beads and were labeled by Alexa Fluor 647-anti-CD31 antibody. These monocytes were injected into the malignant PT PDX-bearing mice via tail vein. The mice were treated with maraviroc 10 mg/kg twice daily for 48 hours before injection. After the monocytes were injected into mice for 24 hours, the tumors were harvested for fluorescent intensity detection (**A**), and were embedded into paraffin sections for human CD68 staining (**B**). Scale bar, 20 μ m. CCR5 inhibitor maraviroc suppresses malignant PT PDX growth in mice. Malignant PT PDXs were generated from malignant PTs from 3 patients. Then each first-generation PDX was transplanted into 2 mice for the treatment, resulting in 6 mice with PDX in each treatment cohort. Maraviroc was injected (intraperitoneally daily) or doxorubicin was injected (intraperitoneally weekly) at indicated dose after the tumors were palpable. The growth curves (**C**), tumor weights (**D**), and PDXs when they were harvested (**E**) are shown as means \pm SD of 6 mice in each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **F**, Maraviroc treatment prevents recruitment of the mouse macrophage (F480 staining) by PT tumors and inhibits the proliferation of the tumor cells, as visualized by Ki67 staining. Scale bar, 40 μ m. **G**, The working model of the positive feedback loop consisting of CCL5-CCR5 axis and CCL18-TIPNMI3 axis between myfibroblasts and TAM that promotes malignant PT tumorigenesis.

malignance of both myofibroblasts and TAMs in breast PTs (Fig. 6G).

TAM polarization can be induced by activation or inactivation of several pathways. The activation of STAT3 has been correlated with the polarization toward the M2 phenotype (40). The over-activation of STAT3 signaling has been linked with the immunosuppressive activities of TAMs and poor outcome of breast cancers and glioblastoma (41, 42). However, in our study, STAT3 was inactivated upon CCL5 treatment and recovered its activity by the CCR5 inhibitor. This was in accordance with the observation that CCR5 inhibitor induced the phosphorylation of STAT3 in the TAMs from liver metastasis of the clone cancers (43). In addition, we found the AKT signaling in the TAMs were activated by the CCL5-CCR5 axis. Activation of PI3K-AKT pathway has been correlated with both M1 and M2 polarization (44). PI3K-AKT pathway provides the key signal to restrict proinflammatory and antiinflammatory responses in TLR-stimulated macrophages (45, 46). On the other hand, activation of PI3K-AKT pathway is also an essential step toward M2 activation of macrophages (31, 32, 47, 48). We found that abrogating the AKT activation by the inhibitor, MK2206, prevented the CCL5- or malignant PT cell-induced TAM polarization, suggesting the activation of AKT signaling is essential for the CCL5-induced polarization toward TMA.

Local recurrence rate of PTs remains as high as 8% to 36%, even after surgical resection (49). In addition, the recurrent PTs usually turn more aggressive (50). Given that chemotherapy or radiotherapy is not effective against PTs (2), there is a pressing requirement to develop new therapeutic strategy. CCR5 is predominantly expressed on T cells, macrophages, dendritic cells, eosinophils, and microglia (18). Several CCR5 receptor antagonists are used as antiretroviral drug in treatment of HIV infection (51). Given that CCR5 is expressed on the TAMs and blocking the CCL5-CCR5 axis can prevent the TAM polarization, several studies have applied CCR5 inhibitors for cancer treatment in animal models, such as breast and liver cancers (33–35). A recent phase I trial revealed the antimetastatic effect of maraviroc to advanced colorectal cancer patients with liver metastases (43). In our studies, inhibition of CCR5 by maraviroc prevented the malignant PT-induced TAM polarization and suppressed the PT tumor growth. Furthermore, migration and invasion abilities of PT cells cocultured with TAMs were reduced *in vitro* by blocking the CCL5-CCR5 axis, which was consistent with the fact that lung and liver metastasis of the PT tumors were also inhibited by the treatment of maraviroc. In the PDX tissue of malignant PTs, we found the infiltration of a large population of mouse macrophage. Therefore, the PDXs of malignant PTs were also used to evaluate the therapeutic effect of

maraviroc. We found that maraviroc treatment dramatically reduced the frequency of tumor formation and the tumor growth of malignant PT PDX. Together, our data suggest that the intercellular communication between TAMs and myofibroblasts via the positive feedback loop of CCL5-CCR5 axis and CCL18 and PIPTNM3 axis plays a central role in the tumorigenesis of PTs. Monitoring CCL5 level and targeting CCR5 raise the possibility of precision diagnosis and treatment for breast PTs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Nie

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