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Original Article

Focal adhesion kinase (FAK) deficiency in mononuclear phagocytes alters murine breast tumor progression

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Abstract: While it has long been recognized that mononuclear phagocytes play a significant role in determining breast tumor progression, the molecular factors that contribute to these events are not fully understood. In this report, we sought to determine whether focal adhesion kinase (FAK) expression in this cell population influences primary breast tumor initiation and growth. Using the MMTV-polyoma middle T (PyVmT) murine model of spontaneous breast cancer, we found that FAK expression in mononuclear phagocytes accelerates tumor initiation/progression during the early stages of PyVmT tumor growth but subsequently restricts tumor growth once the tumors have transitioned to malignancy. Mononuclear phagocytes accumulated at the site of developing tumors in a FAK-independent manner. However, once in the tumor, our data suggest that FAK expression is upregulated in the tumor-associated myeloid cells, and its activity in this population of cells may influence the immune landscape of the tumor by supporting the recruitment and/or survival of NK cells. Together, these data support a model in which FAK expression in the mononuclear phagocyte compartment positively regulates the early steps of tumor progression but subsequently functions to restrict tumor growth as the tumors transition to invasive carcinoma.

Keywords: FAK, mononuclear phagocyte, breast cancer, tumor-associated macrophages, MMTV-polyoma middle T

Introduction

It is now a well-established paradigm that infiltrating leukocyte populations can profoundly impact disease progression in many cancers. Cells of the monocyte/macrophage lineage in particular comprise a significant portion of breast tumor stroma and influence many aspects of tumor development [1]. In mice, the majority of tumor-resident cells of the mononuclear phagocyte system (MPS) derive from circulating Ly6C+ monocytes that are recruited from the periphery by a diverse array of tumor-secreted factors [2-4]. Their migration and function are subsequently determined by the stimuli they encounter in the tumor microenvironment. Evidence suggests that monocyte-derived cells are capable of either inhibiting or supporting primary tumor growth [5, 6]. For example, several reports have described direct and indirect anti-tumor responses by macrophages during the early stages of neoplastic development [7-10]. In contrast, a strong correlation between mononuclear phagocyte abundance and poor clinical outcome has been described in advanced breast carcinomas [1], and numerous studies in murine models indicate that these cells ultimately help to establish an environment conducive to tumor progression and metastasis [11, 12]. In these studies, the localization of tumor-associated macrophages (TAMs) to discrete microanatomical regions within the tumor conferred specific functional attributes to these cells, resulting in striking functional heterogeneity between subpopulations of TAMs present within the same tumor [5, 13, 14]. In this way, local cues can direct TAM activation states toward pro- or anti-inflammatory phenotypes that ultimately impact tumor growth.

In order to respond to the heterogeneity within the tumor microenvironment, MPS cells must be equipped to properly integrate the extracellular signals they encounter, which include stromal cells, growth factors, cytokines, and com-

ponents of the extracellular matrix (ECM). Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase that functions as a central regulator of adhesion signaling and motility in many cell types, including macrophages [15, 16]. FAK is recruited to focal complexes and mediates signal transduction downstream of integrin engagement with the ECM or growth factor receptor activation [17]. Through its close relationship with integrins, FAK has also been shown to function as a mechanosensor, transmitting physical cues from the environment that can stimulate cell survival, proliferation, and motility [18-23]. While this molecule has been extensively studied in fibroblasts and transformed cell lines, the function of FAK in MPS populations in the context of breast cancer progression has not been explored. Using the mouse mammary tumor virus polyoma middle T antigen (MMTV-PyVmT) murine model of breast cancer, we therefore sought to determine whether FAK deficiency contributes to mononuclear phagocyte trafficking and/or accumulation in breast tumors, and if tumor progression is influenced by the loss of FAK-dependent activities in MPS cells.

In this report, we provide evidence that FAK-deficiency in mononuclear phagocytes alters the dynamics of tumor development and growth during distinct stages of mammary tumor progression. We show that FAK expression in MPS cells accelerates tumor initiation/progression during the early stages of PyVmT tumor growth. In contrast, FAK expression in the MPS population once the tumors have transitioned to malignancy inhibits primary tumor growth. These changes in tumor growth kinetics are not the result of FAK-dependent trafficking or accumulation of MPS populations at the site of developing tumors. Rather, our data suggest that FAK activity in TAMs may influence the immune landscape of the tumors by supporting the recruitment and/or survival of NK cells, which may ultimately help to control tumor growth and progression.

Materials and methods

Mice

The $FAK^{\Delta myeloid}$ and MMTV-PyVmT mouse models have been previously described [16, 24, 25]. For tumor studies, phenotypically wildtype (WT; $LysM^{wt/wt}$-$FAK^{fl/fl}$) and $FAK^{\Delta myeloid}$ ($LysM^{wt/cre}$-$FAK^{fl/fl}$) mice were crossed with MMTV-$PyVmT^{+/-}$ C57BL/6 mice [25] to produce WT/$PyVmT^{+/-}$ and $FAK^{\Delta myeloid}$/$PyVmT^{+/-}$ mice, respectively. Female WT/$PyVmT^{+/-}$ and $FAK^{\Delta myeloid}$/$PyVmT^{+/-}$ mice (6-18 weeks of age) were age-matched for all analyses. These studies were performed in accordance with the University of Virginia Animal Care and Use Committee guidelines.

Animals were routinely genotyped from tail DNA and subjected to PCR analysis. The following primer sets were utilized: Cre primer set: #1 5'CTTGGGCTGCCAGAATTTCTC, #2 5'TTACAGTCGGCCAGGCTGAC, #3 5'CCCAGAAATGCCAGATTACG (Eurofins Genomics, Louisville, KY). Expected products: WT LysM allele 350 bp, LysM Cre allele 700 bp.

PyVmT target set: #1 5'TGTGCACAGCGTGTATAATCC, #2 5'CAGAATAGGTCGGGTTGCTC (Eurofins Genomics, Louisville, KY). Expected product for $PyVmT^{+/-}$ mice: 200 bp.

Tumor histology

For analyses involving tumor progression prior to the detection of palpable masses, the first thoracic mammary glands (MG1s) were surgically removed with forceps and scissors, fixed overnight (o/n) in 10% formalin, and transferred to 70% ethanol (ETOH). Specimens were embedded in paraffin, sectioned onto glass microscope slides (5 mm sections), and stained with hematoxylin and eosin (H&E). Images were acquired on the Olympus high magnification microscope at 5X magnification. For whole MG1 scans, sections were imaged with a 5X or 10X objective of a Carl Zeiss Axio Imager Z1/Apotome Microscope fitted with motorized focus drives and motorized XYZ microscope stage and stitched together using Stereo Investigator software (MBF Bioscience, Williston, VT). For assessment of tumor progression in pre-palpable mammary tumors, H&E stained sections of MG1s were classified according to distinct stages of PyVmT tumor progression that have been previously described [26, 27]. Investigators were unaware of the genotype of the sample (blinded). To measure the total area of tumor growth in H&E stained MG1 sections, the perimeter of regions containing carcinoma or carcinoma and adenoma/mammary intraepithelial neoplasia (MIN) was traced and the square micron (μm^2) area within the region of interest was calculated using ImageJ software (NIH).

Measurement of tumor growth

For studies assessing the appearance of palpable tumors, MG1s were palpated for tumor growth twice weekly. Once masses were evident, tumors were measured by caliper in two dimensions. Length (l) was defined as maximum attainable tumor measurement in one dimension. Width (w) was defined as the measurement perpendicular to length. Tumor volume was derived using the formula [l x w^2]/2.

Tumor harvest and preparation of single cell suspensions from mouse tissues

Spleens were excised, homogenized between glass microscope slides, filtered through a 30 µm filter, and washed in MACS buffer (0.5% BSA, 250 mM EDTA in PBS). Bone marrow (BM) was flushed from both femora and tibiae with MACS buffer, washed in MACS buffer, and filtered through a 30 µm filter. To remove erythrocytes, the tissues were incubated in ammonium/chloride/potassium (ACK) lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2 EDTA $2H_2O$ in H_2O) for 5 minutes at room temperature and quenched with complete media (10% FBS/DMEM).

Prior to the excision of mammary tissue, vascular perfusion was performed by injecting 10 ml of PBS directly into the left ventricle. Week-10 MG1s were processed as follows: mammary glands were excised, manually minced using scalpel, and then subjected to enzymatic digestion for 60 minutes at 37°C in 0.1 mg/ml Liberase TL (Sigma-Aldrich, St. Louis, MO) and 400 ug/ml DNase I (Roche Biochemical, Indianapolis, IN) dissolved in DMEM (Invitrogen/ThermoFisher Scientific, Carlsbad, CA) under shaking conditions. Red blood cell lysis was then performed, as described above. Samples were subsequently incubated in 0.25% trypsin in DMEM for 2 minutes at room temperature (RT), followed by digestion in 5 mg/ml dispase II (Roche Biochemical, Indianapolis, IN) and 400 ug/ml DNase I for 2 minutes at RT under gentle shaking conditions. Primary carcinomas were processed as follows: tumors were excised, manually minced with a scalpel, and subjected to enzymatic digestion for 60 minutes at 37°C in Accumax (Innovative Cell Technologies Inc., San Diego, CA) under shaking conditions. Red blood cell lysis was then performed, as described above. In all cases, the digested mammary tissue was resuspended in DMEM and strained through a 100 µm filter to remove clumps prior to further processing.

Separation by immunomagnetic column chromatography

CD11b+ myeloid cells were isolated from mouse BM, spleen, and tumor. Tissues were processed and single cell suspensions were prepared as described above. Cell suspensions were washed in MACS buffer and F_C receptors were blocked with α-CD16/32 [1:100] for 10 minutes prior to incubation with α-CD11b conjugated beads (Miltenyi Biotech, Inc., Auburn, CA) for 15 minutes on ice. The suspensions were separated by magnetic columns following the manufacturer's protocol and CD11b+ cells were eluted in MACS buffer for further analysis.

In vitro-derived macrophages from BM

Whole BM was collected as described above. 4-6 × 10^6 cells were added to L929 conditioned media (source of M-CSF) in α-MEM supplemented with 10% FBS and penicillin/streptomycin. At 4 days post-plating, the adherent cells were washed 3 times with PBS, incubated with trypsin/EDTA for 15 minutes at 37°C, quenched with complete media, and washed with PBS prior to further processing.

Immunoblotting

Cell suspensions were washed in PBS, pelleted, and incubated in RIPA buffer for 10 minutes on ice with intermittent vortex [16]. Debris was pelleted by centrifugation (13,000 rpm × 15 minutes) and the supernatants were retained. Protein concentrations were determined by BCA method (Pierce, ThermoFisher Scientific, Waltham, MA) and equivalent amounts of protein were separated by 8% SDS/PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk for 20 minutes prior to adding primary antibodies for overnight incubation at 4°C. α-FAK (Santa Cruz Biotechnology, Dallas, TX) and α-ERK (Cell Signaling Technology, Danvers, MA) were used at [1:1000] in 5% milk. Membranes were washed and secondary antibody [1:5000] was added for 40 minutes at room temperature in 5% milk. The membranes were washed 5 times,

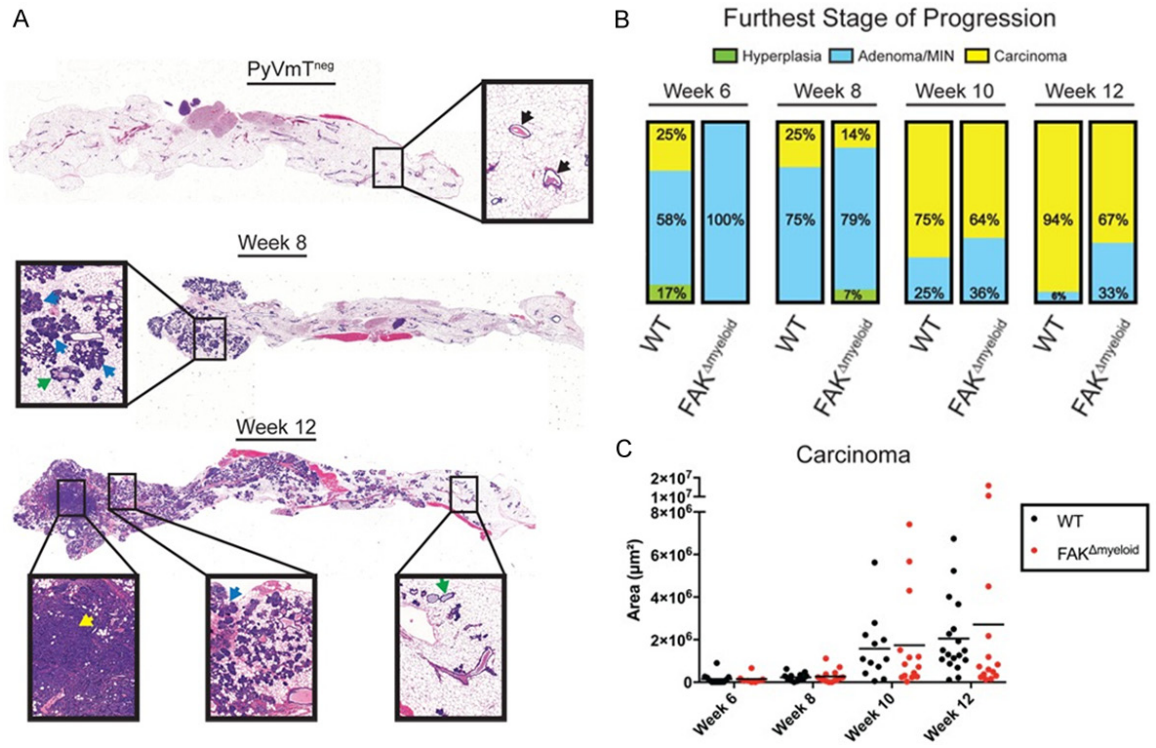

Figure 1. Loss of FAK expression in mononuclear phagocytes delays the early stages of tumor progression. (A) Representative images from hematoxylin and eosin (H&E) stained mammary sections display primary lesions as a function of age in PyVmT mice. Images from MG1s are orientated from nipple proximal (left) to nipple distal (right). Insets display regions of interest at 10X magnification. Arrows designate typical morphology of normal ducts (black), hyperplastic lesions (green), regions of adenoma/MIN (blue), and carcinoma (yellow). (B) Histopathological progression of primary mammary tumors in $FAK^{\Delta myeloid}$/PyVmT and WT/PyVmT MG1s was assessed based on morphological characteristics. Tumor growth was assessed by quantifying the total area (μm^2) of carcinoma. MG1s presenting with $> 2 \times 10^5$ μm^2 of carcinoma were considered to be in the carcinoma stage. Data are presented as the proportional distribution of samples that had reached hyperplasia (green), adenoma/MIN (blue), or carcinoma (yellow) as the furthest stage of disease progression at the indicated ages. Week 6: n=12 for WT/PyVmT, n=8 for $FAK^{\Delta myeloid}$/PyVmT; Week 8: n=12 for WT/PyVmT, n=14 for $FAK^{\Delta myeloid}$/PyVmT; Week 10: n=12 for WT/PyVmT, n=14 for $FAK^{\Delta myeloid}$/PyVmT; Week 12: n=18 for WT/PyVmT, n=12 for $FAK^{\Delta myeloid}$/PyVmT. $P < 0.001$ (Cochran-Mantel-Haenszel test). (C) Total area of carcinoma in MG1 samples isolated from mice at the indicated ages. Each dot represents one MG1.

incubated for 2 minutes in luminol/peroxidase reagents (GE Healthcare, Buckinghamshire, UK), and exposed to film for detection by enhanced chemical luminescence.

Flow cytometry

Single cell suspensions containing approximately 1×10^6 cells in 100 µl MACS buffer were incubated with the F_c blocking antibody anti-CD16/32 (eBioscience, San Diego, CA, USA) for 10 minutes on ice. The cells were subsequently incubated with primary monoclonal antibodies to cell surface antigens for 25 minutes on ice. Antibodies were purchased from the following companies and used at the concentration suggested by the manufacturer: AbD Serotec, Oxford, UK: anti-F4/80 (Cl:A3-1[clone]); Biolegend, San Diego, CA, USA: anti-Ly6C (HK1.4), anti-CD11b (M1/70), anti-Ly6G (1A8), anti-CD192 (SA203G11); eBioscience, San Diego, CA, USA: anti-CD45.2 (104), anti-CD3e (145-2C11), anti-CD49b (DX5), anti-CD19 (MB-19.1). Samples were stained concurrently with fluorescence minus one (FMO) antibody panels. Samples were washed in MACS buffer, and incubated with fixable live/dead cell stain (Invitrogen, Carlsbad, CA, USA) for 30 minutes on ice. Samples were then washed in MACS buffer, fixed for 20 minutes on ice with Cytofix (BD Biosciences, San Jose, CA, USA), and resuspended in MACS buffer. Data were acquired on a Cyan ADP LX (Beckman Coulter, Brea, CA, USA) and analyzed with FlowJo software (Tree Star Inc., v.10, Ashland, OR, USA). Absolute numbers were calculated using Ac-

cucount beads (Spherotech, Lake Forest, IL, USA) according to the manufacturer's instructions.

Statistical analysis

A Cochran-Mantel-Haenszel test was performed to assess potential differences in tumor progression between WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT mice. Comparisons between groups were made using an unpaired two-tailed student t-test, Mann-Whitney test, or log-rank statistic. *P* values ≤ .05 (*) and ≤ .01 (**) were considered statistically significant.

Results

Loss of FAK expression in mononuclear phagocytes delays the early stages of PyVmT tumor progression

In the PyVmT murine model of breast cancer, distinct stages of tumor progression have been identified based on histopathological changes in ductal morphology that reflect the successive advancement of lesions to malignancy [27]. We began our study by characterizing tumor initiation and growth in this model using H&E stained sections of whole mammary glands collected from WT/PyVmT$^{+/-}$ and FAK$^{\Delta myeloid}$/PyVmT$^{+/-}$ mice (hereafter designated as WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT) at defined ages. In accordance with previous reports [27], we first observed the development of pre-malignant lesions around the main milk-collecting duct proximal to the nipple, after which multiple foci began to appear in distal ducts (**Figure 1A**). Compared with PyVmT^{neg} mice (**Figure 1A**, top), ducts in WT PyVmT mice exhibited morphological features of hyperplasia and adenoma/MIN by 8 weeks of age (**Figure 1A** middle, green and blue arrows, respectively), although phenotypically normal ducts could also be observed in regions more distal to the nipple. By 12 weeks of age, the primary tumor had often advanced to the carcinoma stage, while retaining proximal regions of adenoma/MIN (**Figure 1A**, bottom). We noted that the transition to carcinoma preceded our ability to detect these tumors by palpation.

To determine if FAK expression in mononuclear phagocytes influences tumor control during the early stages of initiation/growth, we assessed the furthest observable stage of neoplastic progression from WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT transgenic mice over a time-course that preceded the appearance of palpable tumors. We elected to focus on mammary gland 1 (MG1) for this study to minimize the considerable variability in tumor latency that had previously been reported as a function of anatomical location, with tumors from MG1 appearing significantly earlier than those that derived from MG2-MG4 [25]. Compared with WT/PyVmT mice, tumor progression was significantly delayed in mammary glands harboring FAK-deficient MPS cells (**Figure 1B**). This suggests that FAK expression in mononuclear phagocytes may positively impact tumor initiation and/or progression to carcinoma. However, when we assessed the total area of the MG1 sections that displayed morphological hallmarks of carcinoma, we found that the average size of the carcinomatous regions was similar in WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT tumors during this early, "pre-palpable" stage (**Figure 1C**). This was surprising, given that the initial transition to carcinoma appeared to be delayed in tumors harboring FAK-deficient MPS cells. These data suggested the possibility that FAK activity in MPS cells may have differential effects in these early stages of tumor growth, on the one hand acting to promote the progression of adenoma/MIN to carcinoma, but on the other hand restricting the outgrowth of tumors once they fully transitioned to the carcinoma stage.

Loss of FAK expression in mononuclear phagocytes accelerates the outgrowth of PyVmT breast carcinomas

To further examine the effect of FAK expression in monocytes/TAMs on tumor growth after the transition to carcinoma, we tracked the appearance of palpable masses in WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT MG1s over time. It is important to note that, once the tumors are palpable in this model, histological sections uniformly exhibit regions of advanced carcinoma (data not shown). Significantly fewer FAK$^{\Delta myeloid}$/PyVmT MG1s remained tumor-free compared with WT/PyVmT MG1s at similar ages (**Figure 2A**), with palpable tumors appearing in FAK$^{\Delta myeloid}$/PyVmT MG1s an average of over 1.5 weeks prior to tumors in WT/PyVmT MG1s (**Figure 2B**). In accordance with the accelerated time to tumor appearance, the average tumor

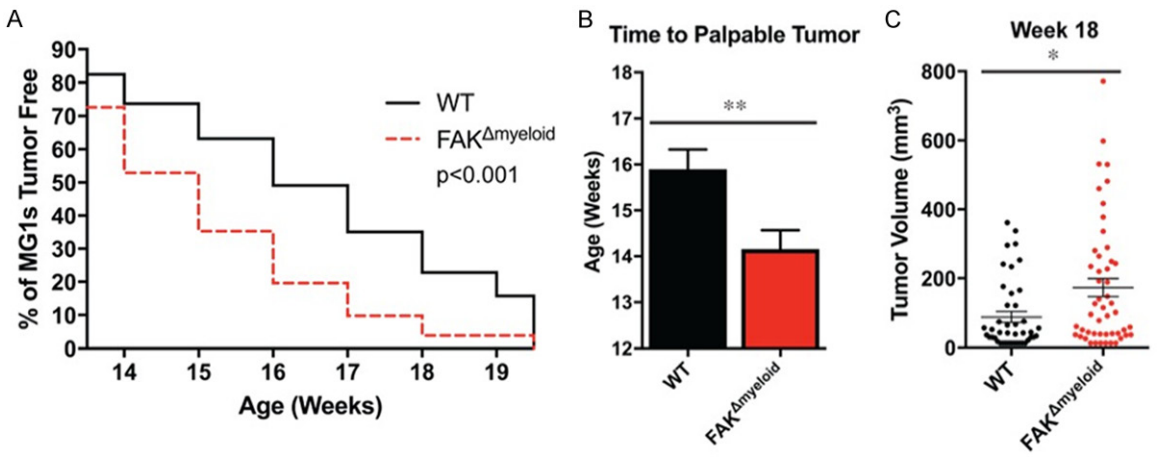

Figure 2. Loss of FAK in mononuclear phagocytes accelerates primary tumor outgrowth. A, B. MG1s in FAK$^{\Delta myeloid}$/PyVmT and control mice were palpated for tumor growth. Data are representative of 57 WT/PyVmT and 51 FAK$^{\Delta myeloid}$/PyVmT MG1s, respectively. A. Kaplan-Meier plot depicts the percentage of mammary glands that remained tumor-free at each week of observation. Data were analyzed by the log rank test. B. The average time to palpable tumor per mammary gland is plotted. **P < 0.01 (Mann-Whitney test). C. MG1 tumor volumes were determined by caliper measurement at week 18. Each dot represents one tumor. *P < 0.05 (Mann-Whitney test). n=43 WT/PyVmT; n=50 FAK$^{\Delta myeloid}$/PyVmT.

volume of primary tumors assessed at week 18 was significantly greater in FAK$^{\Delta myeloid}$/PyVmT than in WT/PyVmT mice (**Figure 2C**). Together, these data suggest that tumor control by MPS populations occurs at least in part through a process that involves FAK.

FAK expression is upregulated in tumor-associated myeloid cells

While FAK protein expression has been characterized in several myeloid cell populations, including macrophages [16, 28-30], FAK expression in tumor-associated myeloid cells has not been assessed. Because primary tumor growth dynamics were altered under conditions of myeloid-specific deletion of FAK, we wanted to determine the relative level of FAK protein expression in tumor-resident compared to peripheral myeloid cells. Lysates were prepared from CD11b+ cells isolated from the bone marrow (BM), spleen, and tumor of WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT mice, and FAK protein expression was subsequently examined by immunoblot (**Figure 3A**). In parallel, lysates were prepared from bone marrow derived macrophages (BMDMs) generated *in vitro*. FAK expression was significantly elevated in tumor-derived myeloid cells and BMDMs compared with myeloid cells isolated from BM and spleen, which failed to express detectable levels of FAK. Notably, BM and spleen have been reported to contain a higher proportion of immature myeloid cells compared with tumor [31]. In support of this conclusion, we found that surface expression of the macrophage marker F4/80 was upregulated in BMDMs and CD11b+ cells isolated from tumor compared with cells isolated from BM and spleen (**Figure 3B**). Together, these data strongly suggest that FAK expression tracks with maturation of immature peripheral myeloid cells to a macrophage phenotype once these cells localize to the tumor.

The accumulation of MPS populations in PyVmT tumors is independent of FAK

Most tumor-resident MPS cells are thought to derive from peripheral myeloid populations [2-4], which as shown in **Figure 3A** express negligible levels of FAK. This would suggest that trafficking of these cells to the tumor is not likely to require FAK. However, since FAK has been shown in a number of cell types to contribute to proliferation and survival signaling [32-35], we next investigated whether FAK deficiency might alter the representation of these cell populations in the tumor microenvironment. To test this hypothesis, MG1s from the stage at which a significant proportion of primary tumors had advanced to the early carcinoma stage (week 10; see **Figure 1B**) were subjected to immunophenotyping by flow cytometry to enumerate monocytes/monocytic myeloid-derived suppressor cells (M-MDSCs) and TAMs (**Figure 4A**). However, no differences in the relative representation of tumor-resident monocytes/M-MDSCs or TAMs were observed

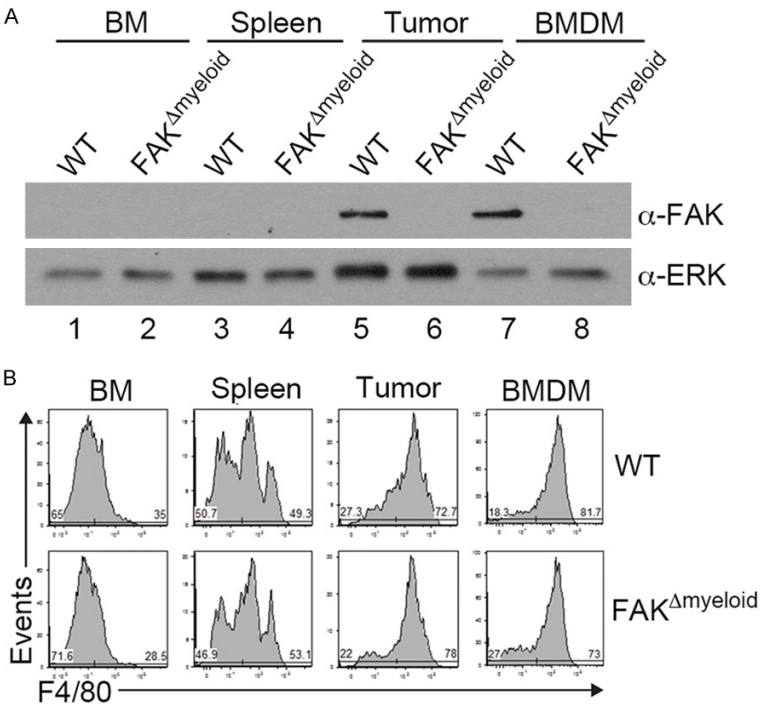

Figure 3. FAK protein expression is upregulated in tumor-associated myeloid cells. CD11b+ cells from the bone marrow, spleen, and tumor of WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT mice were enriched by magnetic bead selection. BMDM samples were generated by culturing whole bone marrow in the presence of M-CSF for 4 days. A. Representative immunoblot showing FAK expression. ERK is presented as a loading control. B. Representative histograms display the percentage of F4/80+ cells among CD11b+ populations isolated from the indicated tissues.

as a function of FAK expression in these cells (**Figure 4B**).

One of the hallmarks of the PyVmT model is that TAM populations accumulate over the course of primary tumor outgrowth [27]. Since changes in the accumulation of these populations could alter tumor growth dynamics, we next compared TAM accumulation in tumors from WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT mice aged 18-19 weeks. Tumors were measured by caliper and binned into "large" and "small" cohorts based on a predetermined tumor volume threshold of 200 mm^3 (**Figure 5A**). Our rationale for this approach was to assess TAM accumulation as a function of tumor size rather than mouse age, as the timing of tumor initiation and disease progression is inherently variable in the PyVmT tumor model. The relative representation and absolute numbers of TAMs (defined as F4/80+ cells) were determined by flow cytometry (**Figure 5B**). As expected, TAM infiltration was significantly greater comparing the WT/PyVmT cohort of large tumors to smaller tumors of the same genotype. This phenotype was also observed in FAK$^{\Delta myeloid}$/PyVmT mice, indicating that the continued accumulation of TAMs in more advanced stages of the disease is a function of tumor size but not of FAK expression in the myeloid population. This is further supported by the finding that there was no significant difference in TAM accumulation when comparing size-matched tumors from WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT mice. Together, these data indicate that FAK does not control the trafficking, accumulation, or differentiation states of MPS cells during the early or late stages of carcinoma.

FAK expression in myeloid cells influences the immune composition of PyVmT tumors

TAMs have been shown to regulate the recruitment and activity of other leukocyte populations, which can also profoundly impact tumor progression [36]. Therefore, we next tested whether the loss of FAK in mononuclear phagocytes resulted in altered immune cell composition of the developing carcinomas. Single cell preparations from WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT MG1s at week 10 were analyzed by flow cytometry for the presence of T cells, B cells, and NK cells (defined as CD3+, CD19+, and CD49b+, respectively) (**Figure 6A**). While no changes in the relative amounts of B cells and T cells were observed, tumors from FAK$^{\Delta myeloid}$/PyVmT mice exhibited a significant reduction in the proportion of NK cells within the tumor-resident leukocyte population compared with WT/PyVmT tumors (**Figure 6B**). This reduction in the representation of tumor-associated NK cells was not due to a global paucity of this cell lineage in FAK$^{\Delta myeloid}$/PyVmT mice, as the relative proportion and absolute numbers of splenic NK cells were similar in WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT mice (**Figure 6C**). These findings suggest FAK activity in mononuclear phagocytes contributes to the recruitment or preservation of NK cell populations in developing breast carcinomas.

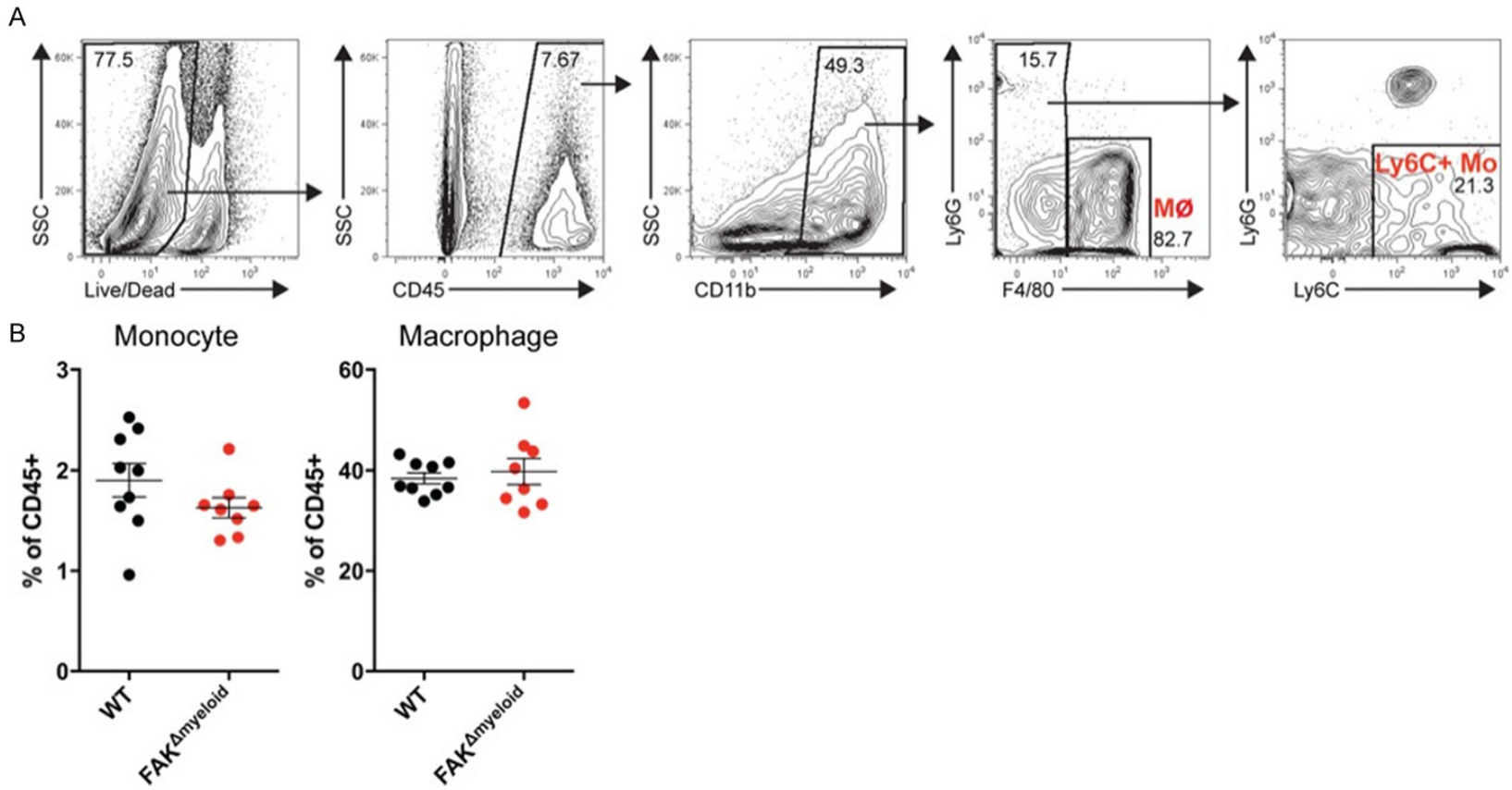

Figure 4. Monocyte and macrophage accumulation in week 10 mammary glands is independent of FAK. A. Representative flow cytometry dot plots depict gating strategies to identify mononuclear phagocyte populations in cell preparations generated from week 10 PyVmT MG1s. B. Percentage of monocytes (left) and macrophages (right) among total CD45+ cells. Each dot represents one MG1.

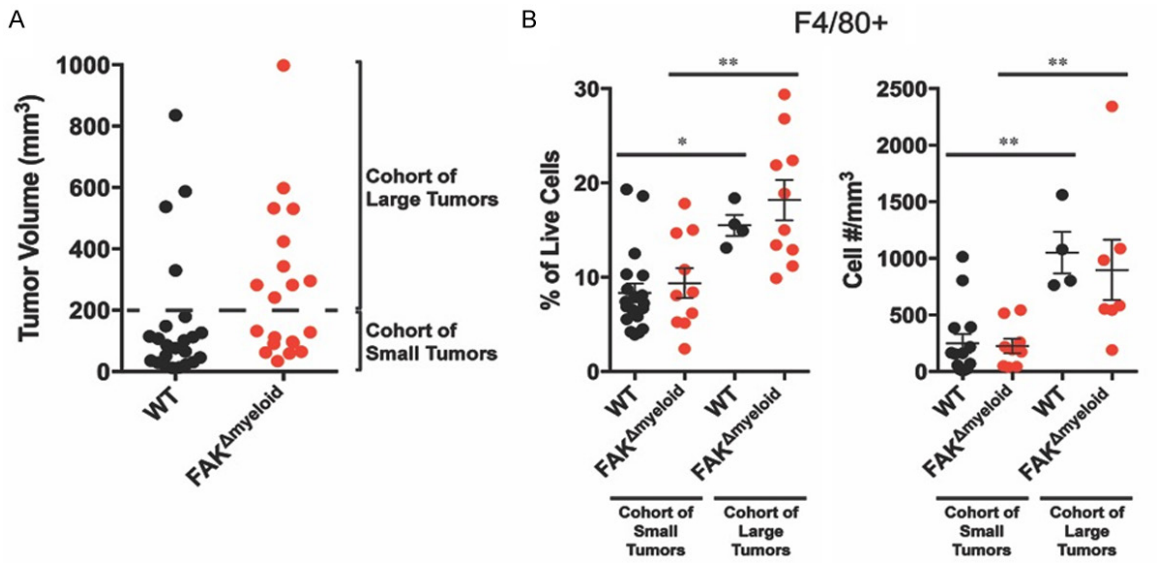

Figure 5. Macrophage numbers in advanced carcinomas correlate with tumor size and are independent of FAK. (A) MG1 tumor volumes from WT/PyVmT and FAK$^{\Delta myeloid}$/PyVmT mice aged 18-19 weeks were determined by caliper measurement. Tumors were binned into cohorts according to size for further analysis. (B) Single cell suspensions generated from the tumors presented in (A) were analyzed by flow cytometry for the percentage of F4/80+ cells among live cells (left), and the absolute number of F4/80+ cells per mm^3 of tumor (right). *P < 0.05, **P < 0.01 (Mann-Whitney test). Each dot represents one MG1.

Discussion

There is considerable evidence that tumor-associated mononuclear phagocyte populations can profoundly impact breast cancer progression based on their ability to either support or inhibit growth and metastasis [37]. The goal of our study was to determine the fundamental contribution of FAK, a critical mediator of adhesion signaling and motility, to macrophage functions regulating breast tumor progression. Using a spontaneous murine model of breast cancer (PyVmT), we demonstrate that loss of FAK expression in MPS cells alters tumor progression during specific stages of the disease. While the initial transition to the carcinoma stage was delayed under conditions of FAK deficiency in myeloid populations (**Figure 1B**), tumor outgrowth immediately after the transition from adenoma/MIN to carcinoma was enhanced under conditions of myeloid-specific deletion of FAK (**Figure 2**). Consistent with its impact on tumor growth, FAK protein expression was shown to be high in tumor-resident MPS cells compared with peripheral myeloid populations (**Figure 3**). FAK did not appear to regulate the trafficking, maintenance, or escalating accumulation of MPS populations during the early or late carcinoma stages (**Figures 4** and **5**). Instead, our data suggest that, once in the tumor microenvironment, tumor-associated NK cells accumulate to a greater extent in the presence of MPS cells that express FAK than when FAK is absent from these cells (**Figure 6**).

Together, these data support a model in which FAK expression in the MPS compartment positively regulates the early steps of tumor progression but subsequently functions to restrict tumor growth as the tumors transition to invasive carcinoma. During the early stages of tumor development, TAMs have been shown to adopt phenotypes in some cases that actively inhibit tumor progression. Our data suggest that FAK serves to provide a counterbalance to the anti-tumor predilection of these cells during these early stages, either by promoting pro-tumor or inhibiting anti-tumor MPS activities. In the absence of FAK, as is the case in FAK$^{\Delta myeloid}$/PyVmT mice, tumor progression becomes restricted because the equilibrium would tip toward the anti-tumor state [8-10]. In contrast, the vast majority of studies attribute TAM activities to a tumor-promoting function during later stages of tumor growth [11, 12]. We suggest that, after the transition to carcinoma, there is a functional switch such that

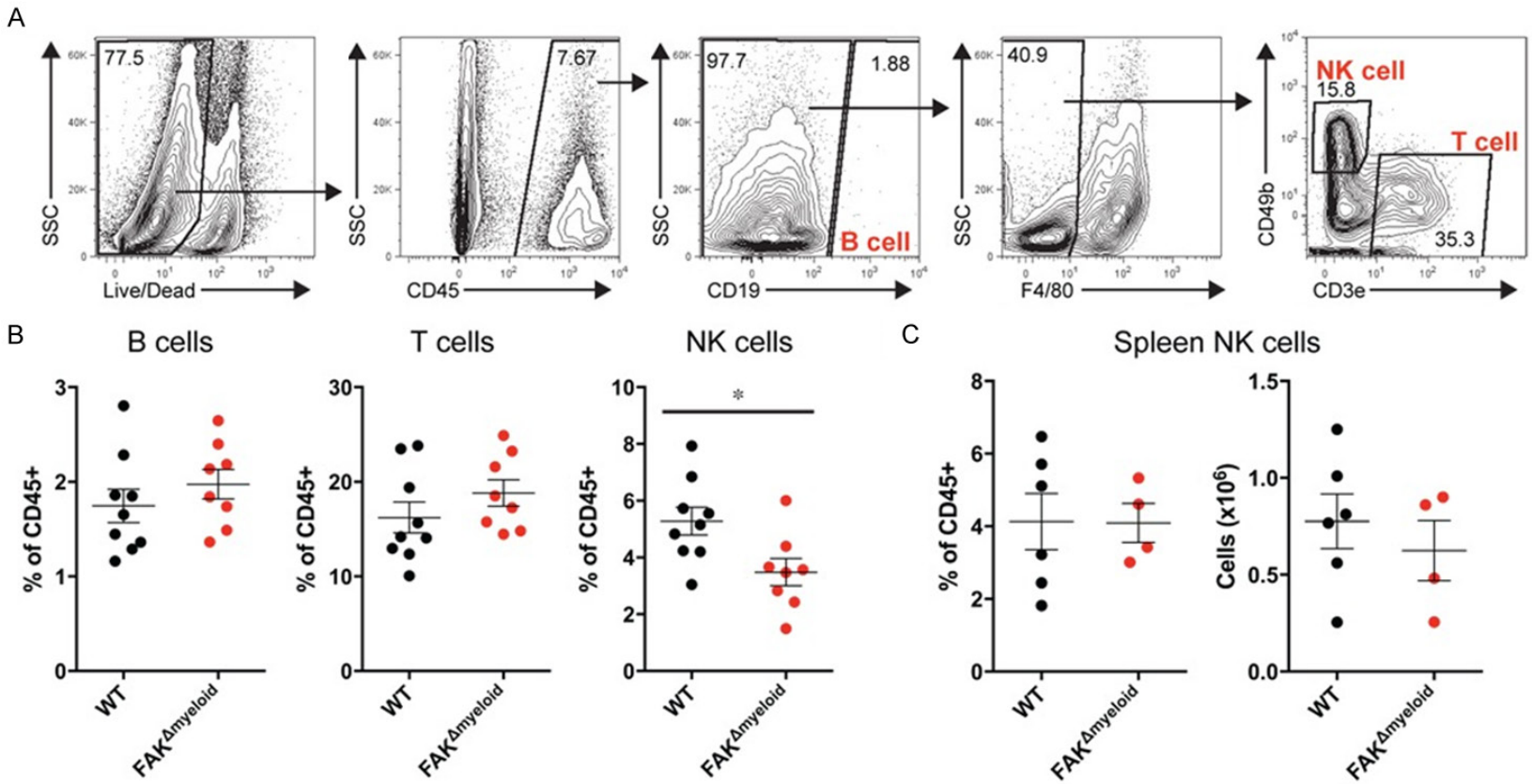

Figure 6. Myeloid-expressed FAK regulates NK cell accumulation in developing tumors. A. Single cell suspensions were generated from week 10 PyVmT MG1s and analyzed by flow cytometry. Representative dot plots depict gating strategies to identify immune cell subpopulations. B. Percentage of B cell lineage (left), T cell lineage (middle), and NK cells (right) among total CD45+ cells. *$P < 0.05$ (unpaired Student's t-test). Each dot represents one MG1. C. Single cell suspensions were generated from the spleen of WT/PyVmT and $FAK^{\Delta myeloid}$/PyVmT mice aged 10 weeks. Samples were analyzed by flow cytometry for the presence of NK cells (identified as $CD45+CD19^{neg}F4/80^{neg}CD49b+$). Graphs display the percentage of NK cells among total CD45+ cells (left), and the absolute number of NK cells/spleen (right). Each dot represents one spleen.

FAK then provides a counterbalance to the pro-tumor activity of TAMs that predominates at this stage. Cells lacking FAK under these conditions would thus exhibit greater tumor-promoting activity, manifested by accelerated tumor growth in the FAK$^{\Delta myeloid}$ setting. We suggest that changes that occur in the tumor microenvironment as the tumor progresses to malignancy are responsible for this switch, particularly since FAK is uniquely positioned to respond to changing environmental cues due to its functions as a mechanosensor and in adhesion signaling.

Using mice with a genetic deletion of M-CSF (*op/op*) that results in the lack of mature macrophages [38], Pollard's group showed that TAMs promote progression to late carcinoma, angiogenesis, and invasion/metastasis in the PyVmT model [26, 39]. In contrast to the data presented herein showing that FAK$^{\Delta myeloid}$/PyVmT mice exhibited more robust growth of late-stage tumors, PyVmT *op/op* mice exhibited a significant delay in the onset of late carcinomas [26]. This disparity indicates that the loss of FAK in mononuclear phagocytes is functionally distinct from a complete lack of TAMs at this stage. One possible explanation for this discrepancy is that PyVmT tumors may require the tumor-promoting activity of TAMs to transition to late carcinoma; tumors lacking TAMs would thus fail to progress to carcinoma as was seen in the *op/op* model. In contrast, our model suggests that depletion of FAK in the context of tumor-promoting TAMs may tip the balance to an even greater tumor-promoting activity, thus resulting in more aggressive carcinoma growth.

TAMs exhibit exceptional functional and phenotypic plasticity that can both positively and negatively influence tumor behaviors [37]. We propose that FAK plays a critical role in this plasticity by integrating mechanical and soluble signals present in the tumor microenvironment, ultimately leading to changes in the recruitment and activity of leukocyte populations, remodeling of the extracellular matrix, and changes in the tumor vasculature as the tumor evolves. In support of this notion, we found that FAK$^{\Delta myeloid}$/PyVmT mice exhibited decreased NK cell representation at the carcinoma stage compared to mice with WT TAMs. Since NK cells can mount an anti-tumor response [40], the reduced number of NK cells present in tumors from the FAK$^{\Delta myeloid}$ mice could contribute to the larger tumor size observed the during the later stages of tumor growth [40]. Similarly, given its role as a mechanosensor and regulator of macrophage motility [16, 19, 34, 41], FAK may control the movement of TAMs within physically distinct microdomains of the tumor. This would be consistent with the elegant studies performed by the Condeelis group using intravital imaging, which underscored the importance of directional migration of these cells within regions of the tumor in close proximity to the vasculature [42, 43].

Altogether, our data support a model in which FAK expression in the MPS compartment positively regulates the early steps of tumor progression but subsequently functions to restrict tumor growth as the tumors transition to invasive carcinoma. Future studies are needed to address the potential contribution of FAK toward macrophage activation states and NK cell recruitment, as well as the molecular mechanisms that could potentially drive these processes.

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Disclosure of conflict of interest

None.

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References

[1] Leek RD and Harris AL. Tumor-associated macrophages in breast cancer. J Mammary Gland Biol Neoplasia 2002; 7: 177-89.

[2] Franklin RA, Liao W, Sarkar A, Kim MV, Bivona MR, Liu K, Pamer EG and Li MO. The cellular and molecular origin of tumor-associated macrophages. Science 2014; 344: 921-5.
[3] Noy R and Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. Immunity 2014; 41: 49-61.
[4] Mantovani A, Allavena P, Sica A and Balkwill F. Cancer-related inflammation. Nature 2008; 454: 436-44.
[5] Gordon S and Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005; 5: 953-64.
[6] Lewis CE and Pollard JW. Distinct role of macrophages in different tumor microenvironments. Cancer Res 2006; 66: 605-12.
[7] Vesely MD, Kershaw MH, Schreiber RD and Smyth MJ. Natural innate and adaptive immunity to cancer. Annual Rev Immunol 2011; 29: 235-71.
[8] Allavena P, Sica A, Solinas G, Porta C and Mantovani A. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. Crit Rev Oncol Hematol 2008; 66: 1-9.
[9] Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nature Rev Cancer 2004; 4: 71-8.
[10] Qian BZ and Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell 2010; 141: 39-51.
[11] Mantovani A, Sozzani S, Locati M, Allavena P and Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002; 23: 549-55.
[12] Sica A and Mantovani A. Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 2012; 122: 787-95.
[13] Lamagna C, Aurrand-Lions M and Imhof BA. Dual role of macrophages in tumor growth and angiogenesis. J Leuk Biol 2006; 80: 705-13.
[14] Medrek C, Ponten F, Jirstrom K and Leandersson K. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. BMC Cancer 2012; 12: 306.
[15] Parsons JT, Slack-Davis J, Tilghman R and Roberts WG. Focal adhesion kinase: targeting adhesion signaling pathways for therapeutic intervention. Clin Cancer Res 2008; 14: 627-32.
[16] Owen KA, Pixley FJ, Thomas KS, Vicente-Manzanares M, Ray BJ, Horwitz AF, Parsons JT, Beggs HE, Stanley ER and Bouton AH. Regulation of lamellipodial persistence, adhesion turnover, and motility in macrophages by focal adhesion kinase. J Cell Biol 2007; 179: 1275-87.
[17] Fraley SI, Feng Y, Krishnamurthy R, Kim DH, Celedon A, Longmore GD and Wirtz D. A distinctive role for focal adhesion proteins in three-dimensional cell motility. Nat Cell Biol 2010; 12: 598-604.
[18] Wang Y, Szretter KJ, Vermi W, Gilfillan S, Rossini C, Cella M, Barrow AD, Diamond MS and Colonna M. IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. Nat Immunol 2012; 13: 753-60.
[19] Tilghman RW and Parsons JT. Focal adhesion kinase as a regulator of cell tension in the progression of cancer. Semin Cancer Biol 2008; 18: 45-52.
[20] Tilghman RW, Cowan CR, Mih JD, Koryakina Y, Gioeli D, Slack-Davis JK, Blackman BR, Tschumperlin DJ and Parsons JT. Matrix rigidity regulates cancer cell growth and cellular phenotype. PLoS One 2010; 5: e12905.
[21] Tilghman RW, Blais EM, Cowan CR, Sherman NE, Grigera PR, Jeffery ED, Fox JW, Blackman BR, Tschumperlin DJ, Papin JA and Parsons JT. Matrix rigidity regulates cancer cell growth by modulating cellular metabolism and protein synthesis. PLoS One 2012; 7: e37231.
[22] Klein EA, Yin L, Kothapalli D, Castagnino P, Byfield FJ, Xu T, Levental I, Hawthorne E, Janmey PA and Assoian RK. Cell-cycle control by physiological matrix elasticity and in vivo tissue stiffening. Current Biol 2009; 19: 1511-8.
[23] Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, Yamauchi M, Gasser DL and Weaver VM. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell 2009; 139: 891-906.
[24] Guy CT, Cardiff RD and Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol 1992; 12: 954-61.
[25] Davie SA, Maglione JE, Manner CK, Young D, Cardiff RD, MacLeod CL and Ellies LG. Effects of FVB/NJ and C57Bl/6J strain backgrounds on mammary tumor phenotype in inducible nitric oxide synthase deficient mice. Transgenic Res 2007; 16: 193-201.
[26] Lin EY, Nguyen AV, Russell RG and Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 2001; 193: 727-40.
[27] Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ and Pollard JW. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. Am J Pathol 2003; 163: 2113-26.

[28] Gotoh A, Miyazawa K, Ohyashiki K, Tauchi T, Boswell HS, Broxmeyer HE and Toyama K. Tyrosine phosphorylation and activation of focal adhesion kinase (p125FAK) by BCR-ABL oncoprotein. Exp Hematol 1995; 23: 1153-9.
[29] Fernandez R, Boxer LA and Suchard SJ. Beta2 integrins are not required for tyrosine phosphorylation of paxillin in human neutrophils. J Immunol 1997; 159: 5568-75.
[30] Kume A, Nishiura H, Suda J and Suda T. Focal adhesion kinase upregulated by granulocyte-macrophage colony-stimulating factor but not by interleukin-3 in differentiating myeloid cells. Blood 1997; 89: 3434-42.
[31] Richards DM, Hettinger J and Feuerer M. Monocytes and macrophages in cancer: development and functions. Cancer Microenviron 2013; 6: 179-91.
[32] Berton G and Lowell CA. Integrin signalling in neutrophils and macrophages. Cellular Signal 1999; 11: 621-35.
[33] Hanks SK, Ryzhova L, Shin NY and Brabek J. Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. Front Biosci 2003; 8: d982-96.
[34] Katsumi A, Orr AW, Tzima E and Schwartz MA. Integrins in mechanotransduction. J Biol Chem 2004; 279: 12001-4.
[35] Subauste MC, Pertz O, Adamson ED, Turner CE, Junger S and Hahn KM. Vinculin modulation of paxillin-FAK interactions regulates ERK to control survival and motility. J Cell Biol 2004; 165: 371-81.
[36] Mills CD, Kincaid K, Alt JM, Heilman MJ and Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol 2000; 164: 6166-73.
[37] Mantovani A, Marchesi F, Malesci A, Laghi L and Allavena P. Tumour-associated macrophages as treatment targets in oncology. Nat Rev Clin Oncol 2017; 14: 399-416.
[38] Wiktor-Jedrzejczak W, Bartocci A, Ferrante AW Jr, Ahmed-Ansari A, Sell KW, Pollard JW and Stanley ER. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. Proc Natl Acad Sci U S A 1990; 87: 4828-32.
[39] Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, Qian H, Xue XN and Pollard JW. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res 2006; 66: 11238-46.
[40] Morvan MG and Lanier LL. NK cells and cancer: you can teach innate cells new tricks. Nat Rev Cancer 2016; 16: 7-19.
[41] Wang HB, Dembo M, Hanks SK and Wang Y. Focal adhesion kinase is involved in mechanosensing during fibroblast migration. Proc Natl Acad Sci U S A 2001; 98: 11295-300.
[42] Sidani M, Wyckoff J, Xue C, Segall JE and Condeelis J. Probing the microenvironment of mammary tumors using multiphoton microscopy. JJ Mammary Bland Biol Neoplasia 2006; 11: 151-63.
[43] Wyckoff JB, Wang Y, Lin EY, Li JF, Goswami S, Stanley ER, Segall JE, Pollard JW and Condeelis J. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Res 2007; 67: 2649-56.