The Protease-Dependent Mesenchymal Migration of Tumor-Associated Macrophages as a Target in Cancer Immunotherapy

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Abstract

Macrophage recruitment is essential for tissue homeostasis but detrimental in most cancers. Tumor-associated macrophages (TAMs) play a key role in cancer progression. Controlling their migration is, thus, potentially therapeutic. It is assumed that macrophages use amoeboid motility in vivo like other leukocytes. However, it has not yet been explored. We examined TAM migration using intravital microscopy in mouse tumors and by monitoring ex vivo tissue infiltration in human surgical samples. We demonstrated that TAMs perform protease-dependent and ROCK-independent mesenchymal migration inside mouse fibrosarcoma and breast cancer explants using their own matrix metalloproteases (MMP). In contrast, macrophages use ROCK-dependent and protease-independent amoeboid migration inside inflamed ear derma and in connective tissue at the tumor periphery. We also showed that inhibition of mesenchymal migration correlates with decreased TAM recruitment and tumor growth. In conclusion, this study elucidates how macrophages migrate in vivo, and it reveals that the MMP-dependent migration mode of TAMs provides a rationale for a new strategy in cancer immunotherapy: to target TAMs specifically through their motility. Cancer Immunol Res; 6(11); 1337–51. ©2018 AACR.

Introduction

Migration is a crucial function of macrophages to maintain tissue homeostasis, defense against infections, and tissue repair. It is also associated with progression of several pathologies, including inflammation and cancer. Macrophages, mainly originating from blood monocytes (1), are recruited in the tumor stroma at all stages of cancer progression (2, 3). Tumor-associated macrophages (TAMs) can represent more than 50% of the tumor cell mass, and their percentage positively correlates with poor prognosis in most cancers (4, 5). TAMs release inflammatory cytokines, which generate a chronic inflammatory environment favoring tumor growth (2, 4–6), and promote angiogenesis, lymphangiogenesis, immunosuppression, tumor cell invasiveness, resistance to therapy, and metastasis (2, 4, 5). Both clinical studies and experiments in mouse models show that TAMs are polarized by the local tumor environment, adopting a protumoral phenotype (4, 7). Through remodeling of the extracellular matrix (ECM), TAMs support tumor cell invasiveness (8) and contribute to metastasis by escorting tumor cells and priming the premetastatic site (9–11). For these reasons, TAMs constitute a new wave of immune-oncology targets (12–14).

Several strategies have been designed to limit TAM recruitment in tumors, including inhibition of growth factors and chemokines (6, 12–18). Although promising, these strategies do not specifically target TAMs but also other macrophage populations, regardless of their beneficial or detrimental role. Specifically, limiting macrophage migration in tumors would be an optimal strategy. However, the poor understanding of the mechanisms of macrophage migration in vivo hinders progress in the field.

Two migration modes have been described in three-dimensional (3D) environments: amoeboid and mesenchymal. The general definition of amoeboid movement is rounded, ellipsoid, or moderately elongated cells that form blebs or generate small actin-rich filopodia (19–21). These cells do not adhere to the ECM but, rather, use a propulsive, pushing migration mode (20, 22, 23). This is a rapid and nondirectional motility involving acto-myosin contraction dependent on the Rho–ROCK pathway. Mesenchymal migration consists of cells presenting a very elongated morphology with long membrane protrusions rich in F-actin (19, 21). The movement is slow and directional, involves cell adhesion on the substratum, and requires proteases to degrade the ECM creating paths through dense environments. It is not inhibited but, rather, stimulated by treatment with ROCK inhibitors (24).

The in vivo migration of lymphocytes, neutrophils, or dendritic cells (DC) involves the ROCK-dependent, protease-independent amoeboid mode (25–28), whereas the mechanisms used by macrophages to migrate in vivo is still unknown. It has been shown in vitro that, in contrast to other leukocytes (25) but similarly to tumor cells (19, 21), macrophages can adopt the two...
mechanistically distinct migration modes (8, 24, 25, 29–33), depending on the ECM architecture (32). Therefore, we investigated the migration mechanisms used by macrophages that are recruited to inflamed tissues or tumors.

By using intravital two-photon microscopy and developing an ex vivo model of macrophage tissue infiltration, we demonstrated both in mouse tumors and breast cancer explants that TAMs use the protease-dependent mesenchymal migration mode, whereas macrophages preferentially use the amoeboid migration to move in peritumorous tissue or inflamed ear derma. Finally, we showed that the inhibition of macrophage mesenchymal migration correlates with a decrease in macrophage infiltration into tumors and an inhibition of tumor growth.

Materials and Methods

Animal treatment

MacBlue (C57Bl/Gal4VP16/UAS-ECFP) mice were obtained from David A. Hume (Roslin Institute, Midlothian, Scotland; ref. 34). Eight- to 12-week-old mice were used in all experiments. Macrophages were imaged by intravital microscopy either in inflamed ear derma or inside/around the tumor on mice equipped with dorsal window chambers, as described in the sections below. When indicated, BB-94 (a broad-spectrum matrix metalloprotease inhibitor; Sigma-Aldrich) or Y-27632 (a ROCK inhibitor; Euromedex) was injected intraperitoneally. A single injection was performed 1 day after ear irritation or 6 days (BB-94: 30 mg/kg and Y-27632: 60 mg/kg) after dorsal window chamber surgery and tumor induction. Second injection of BB-94 (BB-94: 15 mg/kg) was done at day 7 in the tumor model.

For intravital experiments performed with dextran (MW = 70, 2,000 kDa), 100 μL of rhodamine-conjugated dextran solution at 10 mg/mL (Thermo Fisher Scientific) was injected retro-orbitally 24 hours prior to imaging. The half time of clearance from blood is 4 to 5 hours (35). For experiments on BB-94 chronic treatment, C57BL/6 mice (Janvier LABS) were used and treated as follows. First, fibrosarcomas were generated by subcutaneously injecting 10^7 LPB-GFP cells into the mouse flank. The LPB cell line is a highly tumorigenic murine clonal derivative of TBL.CI2, a methylcholanthrene-induced C57BL/6 mouse sarcoma cell line (36). The LPB-GFP cell line was obtained by transduction of LPB cells with an AAV-ecFP retroviral vector encoding the eCFP under the control of long terminal repeat as described (37). FACS analysis confirmed that all LPB tumor cells expressed GFP (see the Results section). Second, after 7 days of tumor growth, littermate mice were treated daily with intraperitoneal injection of BB-94 (30 mg/kg) or DMSO (10 μL/mouse) for 12 days. Tumor progression was monitored by fluorescence microscopy (Leica Macrofluor) of GFP tumor cells in animals anesthetized by isoflurane inhalation (Belamont-Neuilly-sur-Seine) throughout the imaging session. Tumor area was automatically quantified using image software. At day 12 of treatment, mice were sacrificed, tumors were resected, fixed, and analyzed by IHC to label TAMs as described in the sections below. All experiments were performed according to animal protocols approved by the Animal Care and Use Committee of the Institute of Pharmacology and Structural Biology (No. 20140315/195).

Macrophage imaging in inflamed ear derma

For macrophage imaging of inflamed ear derma, mouse ears were depilated and imaged before and after irritation. To induce recruitment of macrophages from blood monocytes into the tissue, MacBlue mouse derma was irritated with dinitrofluorobenzene (DNFB) in order to trigger local inflammation. Irritation was performed in two steps: a sensitization step using 10 μL of 0.15% DNFB (Sigma Aldrich) in a 4:1 acetone-olive oil mix, followed by a challenge step 7 days later using the same solution. For imaging purposes, the front of the ear was made to adhere to a plastic base plate and vacuum grease was used to create a well around the back of the ear inside which PBS was added. A glass coverslip was then placed over the ear for imaging.

Dorsal window chamber surgery and tumor induction for intravital experiments

Surgery was carried out under general anesthesia using intraperitoneal injection of ketamine (100 mg/kg; Virbac) and xylazine (16 mg/kg; Bayer). The back of the mouse was shaved and depilated with depilatory cream (Veet), and the eyes were kept hydrated throughout the surgery with Ocry-gel (TVM). The chamber consisted of two symmetrical titanium frames (API Trading Co.). These frames sandwiched an extended double layer of dorsal skin with the use of stainless-steel screws and sutures. After the implantation of the frames, one layer of the skin (12-mm diameter) was surgically removed, and part of the fat and connective tissues from the lower layer were dissected away under a stereomicroscope (Leica Microsystems) to ensure optimal observation. After addition of sterile PBS, a sterile glass coverslip (Glasswarefabrik Karl Hecht) was attached to the open frame with a stainless-steel snap ring to cover the surgical site and provide microscopic access to the skin tissue. Right after the surgery and on the following day, Profenid (10 mg/kg, 50 μL in each thigh; Sanofi-Aventis) was injected intramuscularly to provide analgesia and to prevent inflammation.

Before attachment of the coverslip onto the window frame, 20 μL of DMEM containing 2 × 10^6 LPB-GFP cells (Life Technologies) were injected subcutaneously in the remaining connective tissue of the lower skin layer. Tumor growth was monitored daily by fluorescence microscopy of GFP tumor cells and by assessment of growth using a macroscope (Leica Macrofluor microscope). Six days were needed in order to obtain a 3-mm diameter tumor, when GFP-expressing TAMs and GFP-expressing LPB cells were imaged.

Intravital microscopy and image analysis

Intravital microscopy was carried out on a customized stage for holding mice using a 7MP upright multiphoton microscope (Zeiss) equipped with a 20×/0.1 objective (df = 1.8 mm) and a Chameleon-Ultra II laser (Coherent). Animals were anesthetized by isoflurane inhalation throughout the imaging session. Animal temperature was maintained at 37°C with an Air-Therm-heated environmental chamber and a heating blanket placed under the mouse.

ECFP-fluorescent macrophages from MacBlue mice and collagen by second-harmonic generation were observed using a 435–485 nm BP filter, and LPB-GFP tumor cells were observed using a 500–550 nm filter. The tumor mass, the peritumorous area of the window, or the inflamed ear derma was positioned under the objective and imaged with the laser set at a 940 nm excitation wavelength. A 3D-image stack of sections with z-spacing of 2 μm was acquired every 3 minutes for 1 to 2 hours to assess 3D cell motility in dynamic z-stack time series.
Imaris (BitPlane) software was used to process acquired images, to track cells, and to measure cell roundness. Cell tracking was performed manually using the spots function of Imaris. For each image, 100 randomly chosen cells were tracked for at least 30 minutes. The roundness of tracked cells was obtained by calculating the average value of the width over length ratio at three time points (start, middle, and end). Width was taken at the cell’s mid-length. Macrophages adopted a large variety of shapes from round (roundness of 1) to very elongated (roundness of 0.1).

Isolation and differentiation of human monocyte–derived macrophages

Human monocytes were isolated from the blood of healthy donors (buffy coat obtained from Etablissement Français du Sang) and differentiated into human monocyte–derived macrophages (hMDMs) as previously described (32). Blood samples were obtained following standard ethical procedures and with the approval of the concerned internal review boards.

Isolation and culture of murine bone marrow–derived macrophages (BMDMs)

Bone marrow cells were isolated from femurs and tibias, cultured, and differentiated as described (29).

Patients and tissue surgical samples

Breast tumors were obtained from 23 patients at the University Cancer Institute–Toulouse Oncopole. In this study, tumors larger than 1 cm diameter from patients with breast tumors were included with no exclusion criteria. Samples were collected after surgical tumor resection, placed in a sterile compress soaked with cold RPMI medium for 30 minutes and immediately processed. The clinicopathologic data of the patients are summarized in Supplementary Table S1. Pathologic diagnosis, as well as mitosis status, were confirmed by a referent pathologist in breast cancer. Sample fibrosis was automatically quantified using an image analysis software (Tissue-Studio 4.3, Definiens). This study was approved by the ethic committee (Breast Group of IUCT, transfer license No.: AC-2016-2658; collection statement No.: DC-2016-2656). Patients gave informed written consent for the use of biological samples for research purposes.

Ex vivo tissue slice preparation and coculture with human macrophages

Human and mouse tissue slices were prepared as follows. For mouse samples, tumor induction was performed in C57BL/6 mice identically to induction in dorsal window chamber-equipped mice. However, tumor growth was monitored for 2 to 3 weeks in order to obtain an approximately 1-cm³ tissue before resection. Tissue samples were embedded in 3% low gelling temperature agarose (type VII-A, Sigma-Aldrich) prepared in PBS to allow for easier slicing. Slices measuring 500 μm were obtained using an acid-resistant TSE Systems tissue slicer (TSE Systems) filled with ice-cold PBS (Life Technologies) to set medium blade and arm speeds. Slices were cultured on a 30-mm cell culture insert featuring a hydrophilic PTFE membrane (0.4-μm pore size, Merck Millipore) placed inside 6-well plates containing 1.1 mL of RPMI with or without 10 μmol/L BB-94 or 20 μmol/L Y-27632. A 5-mm diameter stainless-steel washer was then placed on top of each tissue slice to create a well for macrophages seeding. The same day, cocultures were performed by seeding 3 × 10³ hMDMs pretreated for 16 hours with DMSO, BB-94, or Y-27632 on top of tumor slices and incubated in a 37°C, 5% CO₂ environment for 72 hours. After 16 hours of coculture, the washer was removed.

For experiments performed with the irreversible inhibitor of MMP2, -9, and -14 (39), hMDMs were pretreated with 10 μmol/L of MMP2/MMP9 inhibitor V, also known as SB-3CT (4-(4-(Methanesulfonylamino)phenoxy)phenylsulfonyl) methylthiirane, Calbiochem), for 16 hours, washed, and then seeded on top of tissue explants for 48 hours. The potential cytotoxic effect of drugs was evaluated by trypan blue exclusion on hMDMs plated on coverslips. None of these drugs induced cell death over the 2 to 3 days of incubation.

For human samples, hMDMs were labeled with cell tracker (C7025-Green-CMFDA, Invitrogen) according to the manufacturer’s instructions prior to seeding on tissue slices (3 × 10³ hMDM per slice). Culture medium (RPMI 1640, Gibco) complemented with indicated inhibitor was replaced daily for 3 days before overnight fixation with formalin at 4°C (Sigma-Aldrich).

The optimal conditions for ex vivo culture of tumor slices were determined according to tissue viability (as evaluated by the absence of necrosis revealed by tissue staining with hematoxylin–eosin).

IHC on tissue slices

Tissue slices used in cocultures with hMDMs were embedded in paraffin. For quantification assays of macrophage infiltration, slices were then cut along the diameter and serial sectioning was performed along the cut. Sections were stained using either a mouse anti-human CD68 (clone KP1, DAKO, 1/100) for mouse tissues or a rabbit anti-fluorescein (Abcam ab6556) for human samples and a peroxidase-coupled secondary antibody. The specificity of the antibody was verified in vitro on nonlabeled or cell-tracker labeled macrophages seeded on glass coverslips. Sections were stained with hematoxylin to visualize cell nuclei. Slides were then scanned using a Pannoramic 250 Flash II slide scanner with Pannoramic viewer software (3DHISTECH) with a 20× magnification lens.

Macrophage infiltration was quantified using a macro developed in ImageJ as described (33). Briefly, colors were deconvolved with Gabriel Landini’s plugin to separate CD68 or Cell tracker staining from hematoxylin coloration. A threshold on specific staining was chosen to dissociate specific signal from the background and applied to every picture acquired from a tumor slide. For Figs. 5–7 and Supplementary Fig. S5, cells were automatically segmented by thresholding hematoxylin-positive nuclei using the autolocal threshold ImageJ function. Cells were identified as macrophages when at least 35% of the segmented area was CD68- or cell tracker-positive. The distance to the upper part of the section (defined manually) was obtained using the Distance Map ImageJ function.

For ECM characterization of LPB tumors, tumors grown for 1 week were used identically to in vivo experiments. After fixation and paraffin embedding, serial tumor slices were cut and stained with Sirius red to label the whole extracellular matrix or with anti-collagen I (ab34710, abcam, 1/500), anti-collagen IV (ab6586, abcam, 1/500), anti-fibronectin (ab5413, abcam, 1/250), and anti-laminin (L9393, Sigma, 1/50). Anti-F4/80 (MCA497, Serotec, 1/100) was used to visualize endogenous TAMs. Sections were counterstained with hematoxylin. For IHC characterization of the polarization of human and mouse macrophages in tissues, rabbit polyclonal anti-iNOS (clone 54; BD Transduction Laboratories),
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anti-Arg1 (clone H52; Santa Cruz), anti-Arg1 (clone N20; Santa Cruz), anti-CD163 (clone 10D6, Novocastra), and anti-CD206 (clone MRC1; Sigma) were used.

**In vitro coculture of hMDMs and LPB tumor cells**

hMDMs labeled with violet vector cell tracker were coincubated in 6-well plates with mouse LPB-GFP tumor cells (one hMDM for 10 tumor cells) for FACs analysis. Cells were harvested after 72 hours of coincubation (time of incubation of human macrophages with mouse tumor explants), and the cell suspension was incubated with Fc block and antibodies: anti-CD206-APC, as a M2 marker, and anti-iNOS-PE575, as a M1 marker, before analysis with an LSR II Flow Cytometer (BD). A scatter plot of SSC versus FSC was first used to discriminate cells on size and dissociate intact cells from cellular debris and cell aggregates. The GFP versus AmCyan/violet plot were then gated to identify the two cell populations from cellular debris and cell aggregates. The GFP versus AmCyan fluorescence signal and macrophages by AmCyan fluorescent signal. The CD206 versus iNOS plot was gated on the AmCyan-positive macrophage population to analyze macrophage polarization status. For immunofluorescence experiments, hMDMs were coincubated with LPG-GFP tumor cells on glass coverslips in 24-well plates (ratio was 1 hMDM per two tumor cells). Cells were fixed after 72 hours and labeled with Texas-red phalloidin to distinguish macrophages (red) from tumor cells (red and green) and to measure macrophage phagocytic activity of tumor cells (red cells with green inclusion) with the confocal microscope.

**Digestion and flow cytometry analysis of tumors and inflamed derma**

Subcutaneous GFP-expressing fibrosarcoma tumors or inflamed dorsal derma were resected from MacBlue mice and from WT mice as a control, cut into small 1-mm³ pieces, and incubated in digestion medium containing Collagenase IV or Collagenase I (0.34 mg/mL; Life Technologies), respectively, in complete RPMI (Life Technologies) for 2 hours at 37° C. Digestion products were then filtered through a cell strainer (BD) and homogenized using the plunger of a 1-mL syringe. The homogenate was resuspended in complete RPMI and centrifuged 10 minutes at 200 g. The pellet was then resuspended and filtered again through a cell strainer before a second round of centrifugation. The cell pellet was resuspended in sorting buffer (1 mmol/L EDTA, 25 mmol/L HEPES at pH 7.0 in PBS; Life Technologies). Cells (5 x 10⁹) in 100 μL buffer were incubated with 1 μL Fc block (BioLegend), 1 μL anti-CD45–PerCP-Cy5.5 (leucocytes marker, BioLegend), 1 μL anti-F4/80–APC-Cy7 (macrophage marker, BioLegend), 1 μL anti-CD11b–APC (Immunotools), 1 μL anti-CD11c–PE-Cy7 (BioLegend), 1 μL anti-Ly6C–PE (BioLegend), and 1 μL anti-MHC-II–FICt (BioLegend) or –AF700 (eBioscience) for 30 minutes at 4°C. Cells were then centrifuged, resuspended in 300 μL sorting buffer, and analyzed with an LSR II Flow Cytometer (BD). A scatter plot of SSC versus FSC was first used to discriminate cells on size and dissociate intact cells from cellular debris and cell aggregates. Leucocytes were then identified among ECFP-positive cells by CD45 expression. ECFP+CD45+ cells were then gated to measure CD11b and F4/80 expression. Finally, the Ly6C versus MHC-II plot was gated on CD11b+F4/80+ cells. For the characterization of F4/80-negative cells, the MHC-II (E), Ly6C (F), and CD11c (G) plots were then gated on F4/80 negative cells.

**Gelatin zymography**

LPB tumor cells (5 x 10⁹) and 10³ macrophages were cocultured overnight on glass coverslips in the presence of DMSO, BB-94 (10 μmol/L), inhibitor V (20 μmol/L), or Y-27632 (20 μmol/L). Conditioned cell supernatants and cell lysates were collected and analyzed by gelatin zymography as described (29). Briefly, samples were subjected to a 10% (w/v) SDS–PAGE electrophoresis that contained gelatin (0.1 mg/mL). The gels were then incubated in 2.5% (v/v) Triton X-100 to remove SDS, then rinsed three times in water. The gels were incubated overnight at 37° C in gelatinase buffer (10 mmol/L CaCl₂, 1 μmol/L ZnCl₂, 100 mmol/L Tris/HCl, pH 7.5), then stained with 0.5% Coomassie Brilliant Blue G-250, followed by destaining in 10% (v/v) acetic acid/30% (v/v) ethanol/5% (v/v) formaldehyde in water. Gelatinolytic activity was detected as unstained bands on a blue background and quantified using ImageJ software.

**Statistical analyses**

Statistical differences were analyzed with two-tailed unpaired Student t tests or two-way ANOVA for in vitro experiments and mouse experiments using GraphPad Prism 6.0 (GraphPad Software Inc.). In all cases, P < 0.05 was considered statistically significant. For human samples, repeated-measures ANOVA was performed to evaluate the effect of treatment on macrophage infiltration. All reported P values were two sided. For all statistical tests, differences were considered significant at the 5% level. Statistical analysis was performed using STATA 12 and R 3.4.0 software. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.

**Results**

Macrophages use the amoeboid migration mode in inflamed derma in vivo

To investigate the migration of macrophages in vivo, we used a transgenic mouse model with ECFP-expressing macrophages (MacBlue; ref. 34). The MacBlue transgene selectively labels monocytes but not resident tissue mononuclear cells, except for microglia and Langerhans cells (40). ECFP expression is maintained in monocyte-derived macrophages recruited in inflamed tissues (40). First, the migration of macrophages was investigated in an inflammatory model of a contact dermatitis induced by DNFB (Fig. 1A). In untreated control ears, only a few motionless Langerhans cells, identifiable by a star-like phenotype, were detected (Fig. 1B, day 0; Supplementary Video S1). Following irritation, we observed a pronounced recruitment of fluorescent and motile cells (Fig. 1B, day 8; Supplementary Video S1) squeezing between collagen fibers (Fig. 1C; Supplementary Video S1). These cells displayed round shapes (Fig. 1B–E; Supplementary Video S1) and moved in a nondirectional manner, with an average speed close to 2 μm/minute (Fig. 1F and G, control), suggesting the use of the amoeboid mode of migration.

The day after the last DNFB challenge, we isolated the fluorescent cells recruited in the inflamed derma and analyzed them by flow cytometry (Supplementary Fig. S1A–S1E). Ly6C and MHC-II markers were used to distinguish Ly6C⁺MHC-II⁺ monocytes from macrophages that were either immature (Ly6C⁺MHC-II⁺) or mature (Ly6C⁻MHC-II⁺) as previously described (41). We observed that about 80% of CD11b+F4/80+ cells were Ly6C⁺ and MHC-II⁺ (Supplementary Fig. S1E and S1F, yellow) and that about 15% were Ly6C⁻ and MHC-II⁺ (Supplementary Fig. S1E and S1F, blue). This showed that a continuum of differentiation...
Proteases Are Required for TAM Migration In Vivo

Figure 1.
Macrophages perform amoeboid migration in inflamed ear derma in vivo. A, Experimental scheme: MacBlue mouse ear inflammation and drug treatment were performed as shown (Y-27632/control n = 4 mice/group; BB-94/control n = 3 mice/group). B–D, Image stills extracted from dynamic z-stack time series (see Supplementary Video S1). B, Macrophage recruitment in the ear derma after DNFB irritation compared with untreated derma. Langerhans cells: red arrows. Scale bar, 10 μm. C, Macrophages (cyan, white arrowheads) moving in-between collagen fibers (magenta) inside inflamed derma. Scale bar, 10 μm. D, Macrophage morphology inside the inflamed ear derma in control or treated conditions. Scale bar, 10 μm. E and F, At least 100 macrophages were analyzed in each condition outlined in A, and boxplots are shown with 10–90 percentile whiskers for three independent experiments. E, Quantification of macrophage roundness (calculated as the width over length ratio) and (F) average speed (μm/minute) in the ear derma in control or treated conditions. Two-tailed unpaired t test. G, 3D map of trajectories of individual macrophages in the ear derma. Tracks from 25 macrophages of one representative mouse are shown. Blue: the start of the track; red: the end of the track.
status from newly recruited monocytes to fully differentiated macrophages may exist, and that most of the F4/80+ cells are either immature or mature macrophages. F4/80+ cells were distributed from the derma to the subcutaneous connective tissue that contained loose collagen fibers (Supplementary Fig. S1G). Both Arg1+ and iNOS+ cells were detected in the inflamed tissue (Supplementary Fig. S1H). Immuno-histo-fluorescence experiments revealed that a large proportion of iNOS+ cells were Arg1-negative, and the remaining 30% were both iNOS+ and Arg1+, indicative of the presence of myeloid-derived suppressor cells (MDSCs). These results indicated that a majority of inflammatory macrophages and some MDSCs are present in the tissue samples.

To confirm whether these macrophages used the amoeboid mode of migration, as suggested by their morphology, we investigated the two defining characteristics of that migration mode—dependence on ROCK activity and independence of matrix metalloproteases (MMP; refs. 8, 24, 32). Therefore, we intraperitoneally injected either Y-27632, a ROCK inhibitor (21, 32), or BB-94, a broad-spectrum MMP inhibitor (42). Mice with inflamed ear dermas were imaged before (control condition) and 1 day after drug injection (Fig. 1A). We observed that Y-27632 treatment significantly modified macrophages morphology because they became more protrusive (Fig. 1D and E; Supplementary Video S1), as previously described in vitro (24, 32), and reduced their motility (Fig. 1F and G). In contrast, BB-94 treatment did not modify macrophage morphology or motility (Fig. 1D–G; Supplementary Video S1). Therefore, in vivo migration of macrophages in the inflamed ear derma was compared with regard to cell morphology, trajectory, and drug responsiveness (32). Altogether, our results showed that macrophages use the amoeboid migration mode in vivo as previously described for other leukocytes (25–28).

In vivo, TAMs use protease-dependent mesenchymal migration to move inside tumors

We previously showed in vitro that the matrix architecture is a critical parameter to dictate the migration mode used by macrophages (32). Because remodeling of the ECM is a feature of most solid tumors (43, 44), we next investigated if the migration of TAMs could differ from the migration of macrophages in nontumoral ear derma. Therefore, we induced amoeboid migration, with regard to cell morphology, trajectory, and drug responsiveness (32). Altogether, our results showed that macrophages use the amoeboid migration mode in vivo as previously described for other leukocytes (25–28).
newly recruited cells. Rhodamine-dextran stains circulating cells, predominantly blood monocytes, which transmigrate into tissue and differentiate into macrophages (47). Using 70 kDa rhodamine-dextran (injected at day 7; Supplementary Fig. S4A and S4B) that leaks from the vasculature into the tumor (35), we could also stain endogenous TAMs. Thus, 70-kDa rhodamine-dextranþ cells can be both monocytes/macrophages neorecruited from blood and endogenous TAMs. To visualize the newly recruited cells inside the tumor at day 12, 2,000 kDa rhodamine-dextran that does not leak in the tumor was injected at day 11 in a separate set of MacBlue mice (Supplementary Fig. S4C and S4D). About 60% of the blue cells inside tumors were 70 kDa dextranþ at day 8 and at day 12 when BB-94 was cleared out of mice (Supplementary Fig. S4E). This indicated that dextranþ macrophages observed at day 8, when BB94 was injected, persisted to day 12. At day 12, about 40% of 2,000 kDa dextranþ cells were detected inside tumors (Supplementary Fig. S4E), indicative of newly recruited monocytes/macrophages. In conclusion, most of the TAMs were long-lasting cells because at day 12, a portion of the TAMs were also present at the time of BB-94 injection at day 8. These results indicate that TAMs recovered the mesenchymal motility after BB-94 elimination, therefore, excluding a toxic effect of the drug on TAMs. Altogether, these results showed that, in contrast to macrophages recruited to the inflamed derma, TAMs are long-lasting, they exhibit an elongated shape, and their migration is dependent on MMPs and activated by ROCK inhibition, thus, displaying characteristics of in vitro macrophage mesenchymal migration (8, 24, 32).

In vivo, macrophages display an amoeboid migration mode in the peritumorous tissue

In the same mice shown in Fig. 3, we imaged the area surrounding the tumor in the window chambers (Fig. 4A, PT). The peritumorous area was characterized by the lack of LPB-GFP cells in
with most being round cells (Fig. 4C, white arrowheads) and a few elongated macrophages (Fig. 4C, yellow arrowheads). When Y-27632 was injected, macrophage motility was inhibited by 66.6% ± 8.0% (mean ± standard error of 100 cells; Fig. 4D and E; Supplementary Video S3), similarly to ear derma. BB-94 treatment also partially affected macrophage average speed with a 31.1% ± 7.5% decrease (mean ± standard error of 100 cells; Fig. 4D and E; Supplementary Video S5). Both Y-27632
Figure 4. Macrophages perform amoeboid migration to migrate into the peritumoral tissue. The same MacBlue mice (n = 6) carrying a subcutaneous tumor in a dorsal window chamber used in Fig. 3 were imaged in the peritumoral area to examine the macrophage migration. A, Sirius red staining of the ECM in tissues harvested from the window chamber after imaging and fixation: (T) tumor, (PT) peritumoral tissue. Scale bars, 500 µm left image and 50 µm right image. B, Roundness and average speed of 200 macrophages untreated mice (n = 4) in the peritumoral area compared with TAMs in the same mice. Boxplots are shown with 10–90 percentile whiskers. Two-tailed unpaired t test. C, Image stills were extracted from a representative time series to illustrate macrophages morphology and motility in the peritumoral tissue. White arrowheads: two examples of macrophages migrating with a round morphology; yellow arrowheads, a macrophage with an elongated morphology. Scale bar, 10 µm. D, 3D map of individual macrophage trajectories in the peritumoral region. Cell tracking was performed on intravital time series. Tracks from 25 macrophages of one representative mouse are shown. Blue: the start of the track; red: the end of the track. E and F, At least 100 peritumoral macrophages were analyzed in each condition, and boxplots are shown with 10–90 percentile whiskers for three independent experiments. E, Quantification of macrophages average speed (µm/minute) and (F) roundness (calculated as the width over length ratio) in the peritumoral area in control (n = 6) or treated conditions (n = 3 mice/group). Two-tailed unpaired t test. G, Macrophage morphology at the tumor periphery in control or treated conditions (stills extracted from Supplementary Video S5). Scale bar, 10 µm.
and BB-94 affected macrophage roundness in opposite ways (Fig. 4F and G). This showed that macrophages used both migration modes, with predominant amoeboid migration in peritumorous derma.

Human macrophage infiltration into breast cancer explants is protease dependent

We next assessed the migration mode used by human macrophages in tumors. We developed an ex vivo migration assay (Fig. 5A) using human macrophages in surgical samples isolated from 23 patients with breast cancer. We chose breast cancer because TAM infiltration has been clearly associated with poor prognosis in these patients (48, 49). Breast cancers were mainly grade 2 and 3 invasive carcinomas of no special type and invasive lobular carcinomas (Supplementary Table S1). Tumoral breast explants were sliced and cultured for 72 hours with cell tracker–labeled hMDMs, without any sign of tissue necrosis (Fig. 5B). Using tissue slices from the same cancer explant, the effects of BB-94 and Y-27632 were explored in parallel to vehicle-treated tissue slices (DMSO). Macrophage infiltration into breast tumor samples was significantly inhibited by BB-94 compared with DMSO ($P = 0.015$; Fig. 5). No significant effect was observed in
Y-27632–treated slices (Fig. 3C). This showed that human macrophages also used the MMP-dependent mesenchymal migration to infiltrate breast tumors.

Intrinsic macrophage MMP activity is instrumental for tumor infiltration

We then assessed whether macrophages use their own proteases to migrate into tumors. Because the surgical samples of breast tumors were too small to perform additional experiments, we used an ex vivo migration assay with hMDMs loaded on explants of mouse fibrosarcomas. hMDMs infiltrated tumor slices, displaying an elongated morphology (Supplementary Fig. S5A, red arrows), and their migration was inhibited by BB-94 and increased by Y-27632 (Supplementary Fig. S5A and S5B). These macrophages were negative for iNOS, CD163, and CD206, suggesting that the daily washes of tumor slices required for addition of fresh inhibitors preclude M1 or M2 polarization (Supplementary Fig. S5C). BB-94 treatment inhibited macrophage MMP activity, whereas Y-27632 had no effect, as evaluated by gelatin zymography (Fig. 6A). Coincubation of human macrophages with mouse LPB tumor cells did not trigger macrophage activation indicated by absence of macrophage polarization and phagocytosis of mouse cells (Fig. 6B and C). Thus, hMDM migration into fibrosarcomas appeared to be similar to that observed in breast cancer explants.

Next, macrophages were treated with the MMP-2/MMP-9 inhibitor V, an irreversible inhibitor of MMP-2, MMP-9, and MT1-MMP (39, 50), and washed before being seeded onto tumor explants. Treatment-induced inhibition of MMP activity was confirmed by gelatin zymography (Fig. 6A). We observed that macrophages lost their ability to infiltrate tumor slices compared with untreated macrophages (Fig. 6D and E), indicating that macrophage tumor infiltration depends mostly on their intrinsic MMPs.

In vivo BB-94 treatment inhibits tumor growth and macrophage accumulation

Studies show increasing evidence that TAMs stimulate tumor development (2, 3, 5), and, thus, we examined whether inhibition of TAM mesenchymal migration would limit TAM infiltration and, therefore, negatively affect tumor growth. LPB fibrosarcomas were generated in C57BL/6 mice, and daily treatment with BB-94 was initiated (Fig. 7A, day 0), using a dose that inhibited TAM motility inside tumors (Fig. 3) without affecting either survival and proliferation of LPB tumor cells nor survival of BMDMs (Supplementary Fig. S2D). Following 12 days of treatment, mice were sacrificed, and the presence of macrophages in tumors was analyzed by IHC (Fig. 7B). A significant decrease of both the percentage of TAMs found in tumors and the size of tumors of treated versus nontreated mice was observed (Fig. 7B and C). This did not correlate with a change in TAM polarization. TAMs remained Arg1+ and iNOS-negative after BB-94 treatment (Fig. 7D). Thus, by inhibiting TAM motility, BB-94 reduced TAM infiltration into tumors, which reduced tumor growth.

Discussion

We report that in vivo macrophage migration involves two modes. In addition to the amoeboid migration generally used by leukocytes (26–28) that we, herein, observed in inflamed ear derma and in the peritumorous tissue, we showed that macrophages can also use the protease-dependent mesenchymal mode in vivo in tumors. We also observed that the macrophage infiltrate human breast cancer tissue explants used the mesenchymal mode, which was made possible through intrinsic MMPs. Chronic treatment of mice with an MMP inhibitor reduced the number of infiltrating TAMs and led to diminished tumor growth.

Leukocytes can migrate in all body tissues to ensure host protection. Although leukocytes are known to use the amoeboid motility, we previously reported that macrophages have the particularity to also use the mesenchymal migration mode in acellular (32) or cellular 3D in vitro environments (8, 25). By modifying the composition and architecture of the 3D ECM, we previously showed that the matrix architecture is the critical parameter determining the migration mode used by macrophages (32). More specifically, we found that the porosity of the matrix is the critical factor: larger pores allow a sliding motility characteristic of the amoeboid mode, whereas lower porosity causes macrophages to use the protease-dependent mesenchymal migration to open paths through which they can progress (30).

Therefore, the use of the mesenchymal mode by macrophages in vivo likely indicates a particular architecture of the tissue. ECM remodeling is a feature of most solid tumors (43, 44). Extensive ECM alterations during tumor progression result in densification of the tissue (51, 52) and, consequently, the stroma progressively stiffens (52), as described for human breast cancers (53). Previous studies show, in vitro and ex vivo, that cells that cannot perform the protease-dependent mesenchymal migration are unable to infiltrate dense matrices (25) or the tumor stroma (e.g., T lymphocytes cannot infiltrate the dense matrix which surrounds tumor islets, ref. 54). Consequently, the reach of the antitumor activities is limited, and the matrix densification may contribute to the immunosuppressive tumor microenvironment (54). As shown here in fibrosarcoma, macrophages mainly migrated in-between tumor cells, where we observed dense ECM, using the MMP-dependent mesenchymal mode. In contrast, MMPs were dispensable in the inflamed ear derma, where the tissue is made of looser ECM. In the peritumorous area, where the ECM is looser than in the tumor, we observed that macrophage migration was mainly amoeboid. However, we observed a partial dependence of macrophage motility to MMPs. This suggested that the tissue might be remodeled due to the proximity of the tumor mass. In human breast cancers, known to have a profound change in ECM organization and an increase in tissue stiffness (53), we observed that macrophages used MMP-dependent migration. Future studies to determine the link between the macrophage migration mode and the biophysical properties of cancer tissues are, thus, needed.

In addition to ECM density, other factors inherent to the tumor environment could trigger the mesenchymal migration of TAMs. We previously showed in vitro that alternatively activated M2 human and mouse macrophages can efficiently use the mesenchymal migration mode in dense matrices, as opposed to classically activated M1 macrophages, which are almost motionless (25). The tumor microenvironment is dominated by Th2-type cytokines, such as IL4 or TGFβ1, which drive macrophages toward an M2-like phenotype (2, 55). For these reasons, it could be hypothesized that tumor-induced polarization of TAMs should promote their infiltration. However, we observed that macrophages migrating into tumor explants using mesenchymal migration were not polarized, suggesting that macrophage polarization status by itself is not sufficient to dictate the choice of the macrophage migration mode in vivo.
Figure 6.
Intrinsic MMP activity is indispensable for macrophage mesenchymal migration. **A**, Human macrophages from three donors were incubated overnight with DMSO, BB-94 (10 μmol/L), the cell-permeable irreversible MMP2/MMP9 inhibitor V (10 μmol/L) or Y-27632 (20 μmol/L). Supernatants of 1.25 × 10^5 macrophages were collected and submitted to a gelatin zymography. One representative gelatin zymography of three is shown. The percentage of MMP activity in supernatants relative to DMSO was calculated from the area of gelatin degradation. **B**, Human macrophages labeled with violet cell tracker were coincubated with mouse LPB-GFP tumor cells (one macrophage for 10 tumor cells). After 72 hours, cells were harvested and analyzed by flow cytometry for CD206 and iNOS expression. A representative gating scheme and graphical summary is shown. Tumor cells were identified by the green fluorescent signal and macrophages by AmCyan fluorescent signal. The CD206 versus iNOS plot was gated on the AmCyan-positive macrophages to analyze macrophage polarization. Results are for 4 independent experiments. **C**, Human macrophages were coincubated with mouse LPB-GFP tumor cells (one macrophage for two tumor cells). Cells were fixed after 72 hours and labeled with Texas-red phalloidin to distinguish macrophages (hMDMs) from tumor cells (LPB-GFP) and to measure macrophage phagocytic activity of tumor cells (red cells with green inclusion) by confocal microscopy. Absence of phagocytic activity is illustrated on the merge pictures. Macrophages are marked by a yellow outline on the right picture. Scale bar: 20 μm. **D** and **E**, DMSO- and inhibitor V–pretreated macrophages were washed, trypsinized, and loaded on tumor explants for 48 hours. IHC analysis was performed as described in Fig. 5, except that anti-CD68 antibody was used to discriminate infiltrating human macrophages from endogenous murine macrophages and quantify tissue infiltration. **D**, Representative pictures of two independent experiments are shown. Scale bar: 20 μm. **E**, The percentage of brown area (CD68^+ cells) over total area was quantified and plotted as a function of invasion depth inside tumor slices. Results are expressed as mean ± SEM of four mice in three independent experiments.
Figure 7.
Chronic treatment with BB-94 reduces TAM recruitment and tumor growth. A, Experimental protocol: Subcutaneous fibrosarcomas were induced by injection of $10^6$ LPB-GFP cells in the flanks of mice ($n = 12$). After 7 days, mice were treated daily with BB-94 (intraperitoneal 30 mg/kg, $n = 6$) or DMSO (10 μL/mice, $n = 6$), and tumors were imaged in anesthetized animals. B, IHC of tumors after 12 days of BB-94 treatment. Serial sections were stained for F4/80 and counterstained with hematoxylin. Representative pictures are shown. Scale bar, 50 μm. Digitized virtual slides were used for quantification of the percentage of macrophages over total cell number inside all nodules of tumors obtained in six mice per group. Boxplots are shown with 10–90 percentile whiskers. Two-tailed unpaired Student t test. C, Colored images of two representative tumors are shown. Scale bar, 1 cm. Images were analyzed using ImageJ software and the ratio of tumor size at the indicated time over tumor size at day 0 was calculated. Results are mean ± SEM. Two-way ANOVA. D, IHC of TAM polarization in LPB fibrosarcomas after 12 days of BB-94 treatment compared with control mice. Serial sections were stained for Arg1 or iNOS and counterstained with hematoxylin. Representative pictures are shown. Scale bars, 50 μm.
Altogether, our findings indicate that macrophages can perform both amoeboid and mesenchymal migration in vivo. This is a unique in vivo property among leukocytes because the migration of lymphocytes, neutrophils, and DCs has been defined as the ROCK-dependent, protease-independent amoeboid mode (25–28). These findings open new perspectives in the field of immunotherapeutic approaches. One can now consider inhibiting TAM mesenchymal migration specifically to limit side effects on other leukocyte-mediated host defenses. We report that chronic treatment with BB-94 reduced tumor growth in mice, not directly by targeting tumor cells, but, rather, indirectly by decreasing the number of recruited TAMs, likely through inhibition of their mesenchymal migration properties. In light of these new data, the use of MMP inhibitors in cancer treatment should be revisited with the aim of targeting TAM migration as soon as a solid tumor is diagnosed and not in patients with advanced stage diseases as performed in the unsuccessful clinical trials in the 1990s (56).

In conclusion, our work establishes that macrophages use two migration modes to move into tissues in vivo and that TAMs can use the mesenchymal mode. This novel migration paradigm could lead to new therapeutic strategies in cancer treatment. Work is in progress to better understand the environmental characteristics and the molecular mechanisms that dictate the macrophage migration mode in both healthy and pathologic tissues and provide further insight into the regulation of macrophage tissue infiltration.

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

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References
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