Visualizing stromal cell dynamics in different tumor microenvironments by spinning disk confocal microscopy

Mikala Egeblad1,*,‡, Andrew J. Ewald1,‡, Hanne A. Askautrud1,2, Morgan L. Truitt1, Bryan E. Welm1,5, Emma Bainbridge1, George Peeters3, Matthew F. Krummel4 and Zena Werb1,*

SUMMARY
The tumor microenvironment consists of stromal cells and extracellular factors that evolve in parallel with carcinoma cells. To gain insights into the activities of stromal cell populations, we developed and applied multicolor imaging techniques to analyze the behavior of these cells within different tumor microenvironments in the same live mouse. We found that regulatory T-lymphocytes (Tregs) migrated in proximity to blood vessels. Dendritic-like cells, myeloid cells and carcinoma-associated fibroblasts all exhibited higher motility in the microenvironment at the tumor periphery than within the tumor mass. Since oxygen levels differ between tumor microenvironments, we tested if acute hypoxia could account for the differences in cell migration. Direct visualization revealed that Tregs ceased migration under acute systemic hypoxia, whereas myeloid cells continued migrating. In the same mouse and microenvironment, we experimentally subdivided the myeloid cell population and revealed that uptake of fluorescent dextran defined a low-motility subpopulation expressing markers of tumor-promoting, alternatively activated macrophages. In contrast, fluorescent anti-Gr1 antibodies marked myeloid cells patrolling inside tumor vessels and in the stroma. Our techniques allow real-time combinatorial analysis of cell populations based on spatial location, gene expression, behavior and cell surface molecules within intact tumors. The techniques are not limited to investigations in cancer, but could give new insights into cell behavior more broadly in development and disease.

INTRODUCTION
Solid tumors contain many different cellular components in addition to tumor cells, including fibroblasts, lymphocytes, dendritic cells, macrophages and other myeloid cells. The tumor microenvironment is defined by these stromal cells, as well as extracellular matrix components (e.g. collagens and fibronectins), growth factors, proteases and even oxygen and metabolites. The composition of the microenvironment varies with tumor stage, and influences both cancer cell and stromal cell functions. Changes in the stromal compartment that occur with increasing tumor stage include alteration of the function of vascular cells and tumor-associated fibroblasts, and increasing influx of inflammatory cells (Bissell and Radisky, 2001; Coussens and Werb, 2002; Lewis and Pollard, 2006).

The different stromal cell types have distinct functions in cancer progression: myeloid cells and fibroblasts can accelerate tumor progression through the recruitment of new vasculature, and through secretion of chemokines, matrix metalloproteinases and growth factors (Egeblad and Werb, 2002; Bhowmick and Moses, 2005; Lewis and Pollard, 2006; Du et al., 2008); dendritic cells can present tumor antigens to activate an anti-tumor immune response (Banchereau and Palucka, 2005); and T-regulatory lymphocytes (Tregs) can downregulate the activity of cytotoxic T-lymphocytes against cancer cells (Colombo and Piconese, 2007). These tumor-associated stromal cells are distinct from their counterparts in normal tissue and are probably heterogeneous in function (Sica and Bronte, 2007).

Pioneering studies have established intravital imaging techniques for analyzing cell dynamics within the tissues of live mice, including within tumors (Brown et al., 2001; Jain et al., 2002; Brown et al., 2003; Halin et al., 2005; Hoffman, 2005; Stroh et al., 2005; Dreher et al., 2006; Sidani et al., 2006; Boissonnas et al., 2007). However, intravital imaging of tumors has been limited by difficulties in contrasting the dynamics of multiple cell types in different tumor microenvironments for extended time periods.

Building on previous efforts, we sought to compare the dynamics of stromal cells between different tumor microenvironments by direct observation. This goal presented several key challenges: (a) the ability to analyze different tumor microenvironments within the same mouse, thereby excluding mouse-to-mouse variation, (b) labeling of different tumor components, (c) a multicolor excitation and detection scheme to follow multiple cell types, (d) optical access to tumors, (e) long-term anesthesia, and finally, (f) fast image collection to minimize motion artifacts. To achieve this goal, we developed and refined a suite of techniques enabling four-color, multi-position, dynamic imaging for extended time periods in different tumor microenvironments within the same live mouse. The application of these techniques has given us new insights into the tumor microenvironment by allowing us to contrast stromal cell behavior between microenvironments. Furthermore, the techniques enabled the subdivision of myeloid cells into distinct subpopulations in vivo, based on their endocytic and migratory activities of stromal cell populations, we developed and applied multicolor imaging techniques to analyze the behavior of these cells within different tumor microenvironments in the same live mouse. We found that regulatory T-lymphocytes (Tregs) migrated in proximity to blood vessels. Dendritic-like cells, myeloid cells and carcinoma-associated fibroblasts all exhibited higher motility in the microenvironment at the tumor periphery than within the tumor mass. Since oxygen levels differ between tumor microenvironments, we tested if acute hypoxia could account for the differences in cell migration. Direct visualization revealed that Tregs ceased migration under acute systemic hypoxia, whereas myeloid cells continued migrating. In the same mouse and microenvironment, we experimentally subdivided the myeloid cell population and revealed that uptake of fluorescent dextran defined a low-motility subpopulation expressing markers of tumor-promoting, alternatively activated macrophages. In contrast, fluorescent anti-Gr1 antibodies marked myeloid cells patrolling inside tumor vessels and in the stroma. Our techniques allow real-time combinatorial analysis of cell populations based on spatial location, gene expression, behavior and cell surface molecules within intact tumors. The techniques are not limited to investigations in cancer, but could give new insights into cell behavior more broadly in development and disease.

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1Department of Anatomy and 2Department of Pathology, University of California, San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143, USA
2Department of Medical Genetics, Ullevål University Hospital and Faculty of Medicine, University of Oslo, Boks 1072 Blindern, NO-0316 Oslo, Norway
3Solamere Technology Group, 1427 Perry Avenue, Salt Lake City, UT 84103, USA
4Present address: Department of Surgery, Huntsman Cancer Institute, University of Utah, 2000 Circle of Hope, Salt Lake City, UT 84112, USA
5Authors for correspondence (e-mail: mikala.egeblad@ucsf.edu; zena.werb@ucsf.edu)

These authors contributed equally to this work
behavior, and their expression of surface markers. Our techniques, together with the use of model organisms, provide a versatile platform for examining the contribution of cell behavior to a range of diseases, including cancer, and infectious and cardiovascular diseases.

RESULTS

Design of a four-color, multi-region, time-lapse, micro-lensed, spinning disk confocal microscope to investigate tumor microenvironments in live mice

To study stromal cell dynamics by live imaging, we developed a suite of techniques: we designed our microscope around a spinning disk confocal scan-head and a high-sensitivity, intensified charge-coupled device (ICCD) camera (supplementary material Fig. S1). This achieved image acquisition times of 17 and 33 milliseconds for $512 \times 512$ and $1024 \times 1024$ pixel images, respectively. We used a robotic stage to allow multi-position, sequential imaging in different tissue microenvironments within the same mouse. Since enhanced cyan fluorescent protein (ECFP) or enhanced green fluorescent protein (EGFP) are most commonly used in reporter mouse lines, we accommodated simultaneous imaging of ECFP and EGFP using excitation selectivity of 405 and 488 nm light, and added 568 and 647 nm excitation for four-color imaging (supplementary material Fig. S2). Within a 12-hour experiment, we typically collected 32,400 images (540 exposures for each of the four colors in three z-planes in five fields). Accordingly, minimizing the moving parts was essential and was achieved using electronic control of the 405 nm solid-state laser, an acousto-optic tunable filter for the argon and krypton lasers, a quadruple pass dichroic and a corresponding excitation clean-up filter.

We gained optical access to the mouse mammary gland by creating a skin flap. Careful monitoring of vital signs and oximetry, and maintenance of normothermia and hydration facilitated mouse anesthesia for a median duration of 7 hours 29 minutes (range: 2 hours 46 minutes-27 hours 29 minutes; $n=69$ mice). It was feasible to image up to 70 μm into the tissue from the first detectable cell layer (supplementary material Fig. S3). Photobleaching of commonly used fluorescent proteins was negligible, as tested by imaging a single field in two colors, 1000 times (supplementary material Fig. S3). Imaging was possible in a well-lit room, aiding monitoring of the live mouse.

Differential behavior of inflammatory cell populations in different tumor microenvironments

Tumor-infiltrating leukocytes include lymphocytes, dendritic cells, myeloid-derived suppressor cells (MDSCs) and macrophages – each cell type having different functions. To investigate the dynamics of tumor-infiltrating leukocytes, we used the $\text{MMTV-PyMT}$ mouse model of mammary carcinoma. This model is progressive (Fig. 1A), with different tumor stages present within the same mammary gland (Lin et al., 2003), and exhibits leukocyte infiltration at all stages (Fig. 1B; supplementary material Fig. S4). We labeled stromal cells through cross-breeding with different mouse lines that each expressed EGFP in different cell populations and at different intensities (Fig. 1D,E,F; Fig. 5). For contrast, we crossedbred mice with the $\text{ACTB-ECFP}$ line to label all cells with ECFP, although the labeling was strongest in the epithelial compartment (Fig. 1C).

Tumor-infiltrating Tregs can downregulate the tumoricidal immune response (Colombo and Piconese, 2007); however, it is not known how the cells behave in intact tumors. We imaged Tregs expressing a fusion-protein of EGFP and the transcription factor Foxp3 (preferentially expressed in Tregs), by crossing $\text{MMTV-PyMT;ACTB-ECFP}$ mice with the $\text{Foxp3^{EGFP}}$ mouse line (Fontenot et al., 2005) (Fig. 1D). Most Tregs were migratory; 55% migrated with an average track length/time of 8 μm/minute (range: 1-14 μm/minute; $n=130$ cells from three mice) and an average displacement of 3 μm/minute (range: 0.3-11 μm/minute; $n=130$ cells from three mice). An additional 14% of the Tregs migrated, but stopped for short intervals (average: 18 minutes; range: 8-46 minutes), typically migrating along, or in proximity to, blood vessels. The median of the furthest distance between a moving Treg and a blood vessel was 20 μm (range: 0-100 μm; $n=98$ cells from five mice), whereas the median of the shortest distance was 5 μm (range: 0-90 μm; $n=98$ cells from five mice; supplementary material Movie 1). Few Tregs infiltrated the tumor mass and of those that did, none migrated. The average velocity and proportions of migrating Tregs, their arrest times and their tendency to migrate along blood vessels were similar to previous reports on tumor-infiltrating cytotoxic T-lymphocytes (Mrass et al., 2006; Boissonnas et al., 2007).

Tumor-infiltrating antigen-presenting cells, including CD11c$^+$ dendritic cells, have the potential of enhancing the immune response to solid tumors (Labbeur et al., 1999). We imaged CD11c$^+$ dendritic-like cells expressing a membrane-targeted fusion-protein of EGFP with the diphtheria toxin receptor (DTR; allowing us to observe the membrane extensions as the cells moved through the tissue) by crossing $\text{MMTV-PyMT;ACTB-ECFP}$ mice with the $\text{CD11c-DTR-EGFP}$ mouse line (Fig. 1E; supplementary material Movie 2). Most dendritic-like cells were migratory, typically at the tumor-stroma border (supplementary material Movies 2,3), although they occasionally stopped and formed longer interactions with cancer cells (supplementary material Movie 2). Dendritic-like cells more commonly infiltrated the tumor mass than Tregs, but those that did were also non-migratory (supplementary material Movie 3). The ability to image CD11c$^+$ dendritic-like cells using spinning disk confocal imaging additionally served as a proof-of-principle that our microscope could detect EGFP-levels barely above the detection limits of fluorescence-activated cell sorting (FACS) (Fig. 1G,H).

Myeloid cells are the most abundant and heterogeneous class of cells that infiltrate tumors (supplementary material Fig. S4). Tumor-promoting myeloid cells fall into two broad groups, the MDSCs and the tumor-associated macrophages (TAMs), and their increased abundance correlates with poor prognosis (Sica and Bronte, 2007). Since the tumor microenvironment may directly influence cell function (Bissell and Radisky, 2001; Lewis and Pollard, 2006), we hypothesized that this would be reflected in observable differences in myeloid cell behavior between microenvironments. To test this hypothesis, we compared the behavior of myeloid cells in normal mammary glands, premalignant hyperplasias and in different areas of carcinomas, by crossing $\text{MMTV-PyMT;ACTB-ECFP}$ mice with the $\text{c-fms-EGFP}$ mouse line, which expresses EGFP under the c-fms promoter (Fig. 2A,A’; supplementary material Movies 4,5). Myeloid cells positive for c-fms were markedly more migratory in the tumor-stroma border.
areas than within the tumor mass, regardless of tumor stage (supplementary material Movie 5). We computed trajectories of individual myeloid cells within different areas, to determine the average displacement over time (Fig. 2A’). In accordance with previous studies (Lindquist et al., 2004), we found that cells with a computed displacement of less than 2 $\mu$m/minutes were virtually non-migratory when observed directly, and that their pseudo-migration was a result of motion artifacts. The tumor-stroma border areas of carcinomas contained the highest fractions of cells with medium (2-4 $\mu$m/minute) and fast (>4 $\mu$m/minute) displacement/time. The lowest fractions of medium and fast migrating cells were found inside the tumor mass at the carcinoma stage (Table 1, $P<0.0001$, chi-squared test). Thus, using multi-position imaging, we found that the myeloid cell population displays heterogeneity, with cell migration varying between tumor microenvironments in the same mouse.

**Differential response of cell migration to acute hypoxia**

Next, we asked whether oxygen tension, a microenvironmental factor, could be responsible for the observed differences in cell migration between microenvironments; for example, lymphocyte migration in explanted lymph nodes is very sensitive to oxygen concentration (Huang et al., 2007). Since oxygen tension varies widely between different parts of tumors (Brown and Wilson, 2004), we hypothesized that differences in myeloid cell migration between microenvironments, in part, represented differences in tissue oxygen tension. We induced acute systemic hypoxia by lowering inhaled oxygen levels from 21% to 7%, but this did not reduce myeloid cell migration acutely (Fig. 2B). In contrast, most Tregs stopped migrating almost immediately upon acute systemic hypoxia (Fig. 2C; supplementary material Movie 6). Interestingly, the Tregs resumed migration when normoxia was re-established (Fig. 2C; supplementary material Movie 6).
Dextran uptake up by a low-migratory, mannose receptor+ macrophage population

Since acute changes in oxygen levels did not influence myeloid cell migration, we determined whether the distinctive behaviors of the myeloid cells in the different microenvironments represented different myeloid cell types (e.g. monocytes, macrophages, granulocytes, dendritic cells and MDSCs). Essentially all c-fms-EGFP-positive cells expressed CD11b (Fig. 3A). Several different subsets of myeloid cells could be recognized in late stage tumors – 68% expressed both CD11b and the macrophage marker F4/80, whereas 47% expressed both CD11b and CD11c [a dendritic cell marker also expressed by some macrophages (Grundy and Sentman, 2005)]. In addition, 13% co-expressed CD11b and Gr1 (Ly-6G), a marker for neutrophils and MDSCs (Mordue and Sibley, 2003; Gabrilovich et al., 2007) (Fig. 3A).

To analyze the behavior of different subsets of c-fms+ myeloid cells within the same mouse and microenvironment simultaneously, we first marked the macrophages with intravenously injected fluorescent dextran (Wyckoff et al., 2007), which over time leaked out of tumor areas and was taken up by a subset of c-fms+ myeloid cells. In the tumor periphery, dextran-ingesting c-fms+ macrophages were non-migratory by direct inspection, even before ingesting dextran, whereas myeloid cells that did not ingest dextran were migratory in these same areas (Fig. 3B; supplementary material Movie 7). We computed trajectories of the total c-fms+ myeloid cell population, as well as from the dextran-ingesting cell population (Fig. 3B).

Table 1. Migratory behavior of myeloid cells varies with the tumor microenvironment

<table>
<thead>
<tr>
<th>Velocity</th>
<th>Normal</th>
<th>Late carcinoma</th>
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<tbody>
<tr>
<td></td>
<td>Stroma (n=385)</td>
<td>Epithelium (n=980)</td>
</tr>
<tr>
<td>Slow: &lt;2 μm/minute</td>
<td>91%</td>
<td>87%</td>
</tr>
<tr>
<td>Medium: 2-4 μm/minute</td>
<td>8%</td>
<td>12%</td>
</tr>
<tr>
<td>Fast: &gt;2 μm/minute</td>
<td>1%</td>
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Velocity of c-fms+ cells were computed from four 1-hour-long time-lapse movies of 200x200 μm fields per microenvironment. Fields with normal epithelium and hyperplasia also contained some of the adjacent stroma. The distribution of myeloid cell velocities differed significantly between the microenvironments (P<0.0001, chi-squared test).
populations using both low (10 kDa) and high (2 MDa) molecular weight dextran (Fig. 3C). Although 19% of the total c-fms+ myeloid cells migrated with a velocity of >2 μm/minute, only 5-6% of the dextran-ingesting macrophages migrated in the same tumor-stroma border areas (Table 2, \( P < 0.0001 \), chi-squared test).

Tumor-infiltrating macrophages can be classified as either the tumoricidal, classical activated/M1 type, or as the pro-tumorigenic, alternatively activated/M2 type, which can stimulate cancer cell proliferation and invasion as well as tumor angiogenesis (Sica and Bronte, 2007). To characterize the macrophages that were marked by dextran, we isolated tumor tissue from dextran-injected mice and stained it for macrophage markers. The c-fms+/dextran+ cells in the tumor-stroma border areas expressed both CD68, a marker of monocytes/macrophages, and CD206 (the mannose receptor), a marker of alternatively activated M2-type macrophages (Mantovani et al., 2002) (Fig. 4A,B). The c-fms+/dextran+ cells in tumor-stroma border areas were mostly CD68+, but negative for CD206 (Fig. 4A,B). Similarly, the c-fms+/dextran+ myeloid cells that had infiltrated the tumor mass (largely sessile) were CD68+, but negative for CD206 (Fig. 4A,B’).

**Analysis of dextran uptake to distinguish non-migratory Fsp1+ macrophages from fibroblasts**

To expand our analysis of the cells within the tumor microenvironment, we looked beyond leukocytes and analyzed the behavior of carcinoma-associated fibroblasts (CAFs) (Fig. 5). Since no single marker exists for CAFs, we used mice that express EGFP, under the fibroblast-specific protein 1 promoter (\( Fsp1^{+/+}, \) EGFP).
mice), in both fibroblasts and macrophages (Inoue et al., 2005), and distinguished between these cell types by the propensity of macrophages to take up injected fluorescent dextran (Wyckoff et al., 2007). We imaged the fibroblasts in the stroma (Fig. 5A), at the tumor border (Fig. 5B) and within the tumor mass (Fig. 5C) from the time of dextran injection and continued imaging for several hours after the injection (supplementary material Movie 8). A small subset of Fsp1+/dextran– cells (enriched for fibroblasts) was migratory, but most cells were non-migratory, regardless of whether they were interspersed in the stroma between fat cells, at the periphery of the carcinoma, or within the carcinoma mass (compare Fig. 5A-C with A'/B'/C'; supplementary material Movie 8). Fsp1+/dextran+ cells (macrophages) were non-migratory.

Patrolling of blood vessels and the tumor-stroma border area by Gr1+ myeloid cells

The numbers of both MDSCs (CD11b+/Gr1– or c-fms+/Gr1+) and TAMs (c-fms+/CD68+) increase with tumor progression (Sica and Bronte, 2007). To analyze the behavior of different subsets of c-fms+ myeloid cells, simultaneously within the same mouse and microenvironment, we labeled two myeloid cell subsets and observed them for over 12 hours. Gr1+ myeloid cells (monocytes, granulocytes and MDSCs) were marked with intravenously injected fluorescent anti-Gr1 antibodies (Chiang et al., 2007), whereas alternatively activated macrophages were marked with intravenously injected fluorescent dextran (Fig. 6A,A'). Within minutes of injecting Alexa Fluor 647-conjugated anti-Gr1 antibodies together with fluorescent dextran, c-fms+ cells with high levels of bound anti-Gr1 antibodies were observed patrolling inside blood vessels, both with and against the blood flow (Fig. 6A',C; supplementary material Movie 9).

We also observed Gr1+ cells extravasating into the tumor stroma (Fig. 6B), and migrating c-fms+/Gr1+ cells at the tumor-stroma border (Fig. 6C) – although these were not as abundant as migrating c-fms+/Gr1– cells. A fluorescently labeled isotype-matched control antibody was injected to control for non-specific antibody uptake or binding by the myeloid cells, but did not label cells in the tumors (supplementary material Fig. S5). The majority of the cells that ingested dextran were c-fms+/Gr1– and sessile. Interestingly, there were also a few non-migratory, c-fms+/Gr1+ cells in the stroma, localized at the tumor-stroma border and along blood vessels (Fig. 6D). Gr1+ cells were not observed inside the tumor.
mass during observation periods of >12 hours. Taken together, these data indicate that MDSC-like cells and TAM-like cells consist of distinct subpopulations that can be distinguished, in situ, in live mice by a combination of transgenic and cell surface markers, tissue location, and cell behavior.

DISCUSSION

The tumor microenvironment evolves with, and influences, tumor progression (Bissell and Radisky, 2001; Coussens and Werb, 2002; Lewis and Pollard, 2006). In this study, we have used long-term spinning disk confocal microscopy to perform a dynamic analysis of the varying stromal cell behavior within the evolving tumor microenvironment, analyzing Tregs, CAFs, macrophages, dendritic-like cells and myeloid cells. Furthermore, we have achieved in situ subdivision of myeloid cells into distinct populations using combinatorial labeling of cells and analysis of location, motility and endocytic behavior (Fig. 6E).

Dynamics of tumor-associated stromal cells in different microenvironments

We showed that tumor-associated stromal cells, including Foxp3+ Tregs, CD11c+ dendritic-like cells, c-fms+ myeloid cells and Fsp1+ fibroblasts, have distinct behaviors. Understanding how these cells behave in tumors may be important for understanding their roles in tumor immunology: Tregs downregulate the activity of cytotoxic T-lymphocytes against cancer cells (Colombo and Piconese, 2007); dendritic cells are used with tumor vaccines (Banchereau and Palucka, 2005); and myeloid cells accelerate tumor progression in mice and are associated with poor prognosis in human breast cancer (Condeelis and Pollard, 2006; de Visser et al., 2006; Lewis and Pollard, 2006). Interestingly, all of the analyzed stromal cell types showed a higher propensity to migrate in the peri-tumor area and lower propensity to migrate within the tumor mass. Possible explanations for the difference in cell migration could be, simply, that the cancer cells within the tumor mass are too tightly attached to each other to enable the further migration of stromal cells. Alternatively, it could be the result of either changes in chemokine concentrations, the presence of factors secreted by the cancer cells to inhibit migration, or the lack of oxygen and metabolites.

In explanted lymph nodes, T lymphocyte migration is very sensitive to changes in oxygen concentrations (Huang et al., 2007). We show that Treg migration in intact peripheral tissue within live mice is sensitive to inhaled oxygen levels. Thus, the sensitivity of lymphocyte migration to oxygen levels is unlikely to be specific to explanted lymph nodes and may explain why these cells often migrate in proximity to blood vessels. Interestingly, the cessation of migration during acute hypoxia was reversible and most Tregs resumed migration after re-establishment of systemic normoxia.

In contrast to Tregs, the myeloid cells showed little sensitivity to induction of acute systemic hypoxia. This was somewhat surprising, since it has been shown that hypoxia inhibits macrophage chemotaxis in transwell migration assays in vitro (Turner et al., 1999; Grimshaw and Balkwill, 2001). However, our observation is consistent with the ability of myeloid cells to infiltrate hypoxic areas in tumors or dermal wounds, where a more chronic exposure to hypoxia may change the ability of the cells to further migrate (Murdoch et al., 2004). The mechanism that makes lymphocyte migration sensitive to hypoxia is unknown, but lymphocytes could have sensors for oxygen or oxygen metabolites [e.g. nitric oxide (NO)], as reported for neurons in Caenorhabditis elegans (Gray et al., 2004). Alternatively, oxygen-dependent metabolism might be essential for migration of lymphocytes but not myeloid cells. The difference in migratory response to acute systemic hypoxia between lymphocytes and myeloid cells raises the possibility that microenvironmental differences in oxygen concentration influence tumor immunology by biasing infiltration of different immune cell populations into regions of tumors with low oxygen levels.

Molecular and cell behavioral subdivision of myeloid cells in intact tumors

Macrophages, which are of the myeloid lineage, have been proposed to respond to different tumor microenvironments by assuming distinct functions (Mantovani et al., 2002; Pollard, 2004; Lewis and Pollard, 2006). Our data suggest that TAMs are a very heterogeneous population; our technology enabled us to identify...
several populations of tumor-infiltrating myeloid cells with distinct behavioral and molecular characteristics. We used static markers on fixed tissue to correlate dextran ingestion, a marker of low migration, with markers of the alternatively activated macrophages. This enabled us to classify c-fms+ myeloid cells into three distinct populations: motile CD68+/CD206–/dextran– cells in the peri-tumor areas; sessile CD68+/CD206+/dextran+ alternatively activated macrophages in the peri-tumor areas; and sessile...
Interestingly, the sessile CD68+ macrophages from within the tumor is probable that receptor-mediated endocytosis is responsible for CD206. Since dextran is a ligand for CD206 (Sallusto et al., 1995), macrophages also lacked CD206.

Because of high hydrostatic pressure, these did not take up dextran. Although dextran may not have penetrated mass were distinct from those at the tumor-stroma border, as they did not take up dextran. Although dextran may not have penetrated into the tumor owing to high hydrostatic pressure, these macrophages also lacked CD206.

**Imaging technology for long-term, multicolor imaging of cell behavior in tumor microenvironments in vivo**

Multicolor intravital imaging techniques have been developed to study tumor-host interactions in real time (Yang et al., 2003; Hoffman, 2005; Yamauchi et al., 2005; Bouvet et al., 2006; Hoffman and Yang, 2006; Yang et al., 2007). However, it has been difficult to compare and contrast cell behavior in different tumor regions, and to image both acute (minutes) and long-term (>12 hours) cell behavior. We developed a novel combination of improved long-term anesthesia protocols, multi-region imaging, and combinatorial fluorescent labeling for imaging in live mice. We used these techniques with micro-lensed spinning disk confocal microscopy, but they can also be used individually or coordinately with other imaging modalities. Our techniques allowed us to compare cell dynamics between cell populations and microenvironments in the same mouse, with all systemic variables kept equal for >12 hours.

We designed our microscope to accommodate the standard transgenic mouse lines and imaging reagents, even when doing so required solving optical challenges. For example, imaging of ECFP and EGFP is non-trivial, but important, since EGFP and ECFP reporter mice are more common than red fluorescent reporter mice. Furthermore, many useful reporters are not very bright, necessitating high sensitivity imaging. We demonstrated this capability by imaging CD11c+ cells, which express EGFP barely above the detection limit by FACS.

Our microscope system accommodates widely varied experimental designs from simple high-speed experiments (60 and 90 frames per second at resolutions of 512x512 and 240x240 pixels, respectively) to long-term, multi-position imaging in four colors and three dimensions over an entire day. Four lines of excitation give great flexibility in labeling techniques, demonstrated here by combining fluorescent proteins, fluorescent dextrans and FACS-validated antibodies. Other major advantages of our imaging system are the large image size (676 x 676 μm) at which we can acquire good resolution (0.66 μm/pixel), and the ability to image in well-lit rooms, aiding the monitoring of the mice.

Multi-photon microscopy can achieve deeper tissue penetration than spinning disk confocal microscopy (Wyckoff et al., 2007) and can image collagen through second harmonic generation (Brown et al., 2003). However, it is less flexible for multicolor excitation and requires imaging in darkness, making long-term monitoring of anesthetized mice more challenging. Thus, our imaging techniques complement other available approaches (Jain et al., 2002; Halin et al., 2005; Hoffman, 2005; Hoffman and Yang, 2006; Boissonnas et al., 2007; Wyckoff et al., 2007).

**CONCLUSIONS AND PERSPECTIVES**

Tumor biopsies have provided insights into the contribution of stromal cells for tumor progression, but cannot inform us about the dynamic behavior of these cells. To investigate the influence of the tumor microenvironment on cell behavior experimentally, we used the progressive MMTV-PyMT mouse model of cancer and observed cell behavior in different tumor regions. Moreover, we demonstrated the feasibility of directly manipulating the microenvironment in real time by changing oxygen levels. More sophisticated manipulations of microenvironmental factors should be possible in the future using genetic, transplantation and pharmacological approaches alone, or in combination.

The MMTV-PyMT model shares molecular and histological characteristics with human epidermal growth factor receptor 2 (HER2)-positive breast cancer (Lin et al., 2003; Herschkowitz et al., 2007). However, whether cells in human tumors behave similarly remains an open question, because no comparable data exist. Techniques are being developed for single-color endoscopic imaging in humans (Hoffman et al., 2006), but four-color, long-term tumor imaging is currently not feasible. Thus, establishing static markers of cellular behavior (such as dextran ingestion) will be necessary to confirm the human relevance of findings on cellular dynamics in mice.

We identified different behaviors of tumor-associated stromal cells in different tumor microenvironments, establishing the microenvironment as an experimental parameter that can be studied in real time, in live mice (supplementary material Movies 3,5,8). In addition, we subdivided the stromal c-fms+ cell population into spatially, behaviorally and functionally distinct subpopulations. The tumor-promoting functions of two myeloid cell populations, the Gr1+ cells and the macrophages, have been shown previously in experiments that have ablated Gr1+ cells (Nozawa et al., 2006) or genetically restricted the numbers of monocytes/macrophages (Lin et al., 2001; Lin et al., 2006). However, these myeloid cell populations clearly display behavioral heterogeneity.

Our results have begun to address the challenge of separating out the functions and behaviors of the different tumor-associated host cell subpopulations, through the process of visualizing the tumor as a living tissue with multiple cellular components. Further experiments that combine pharmacological inhibition or genetic ablation with imaging technologies will complete the link of cell...
behavior with function in tumor progression. The techniques are obviously not limited to the study of cancer, and will facilitate a new understanding of how cells function in their native microenvironments in development and disease.

METHODS

Mice and injections

Mice were crosses between the following strains: MMTV-PyMT and/or ACTB-ECFP (both from Jackson laboratory), and Foxp3\textsuperscript{EGFP}, CD11c-DTR-EGFP, c-fms-EGFP, Fsp1\textsuperscript{+/EGFP} or Sca1\textsuperscript{EGFP} (Guy et al., 1992; Hadjantonakis et al., 2002; Jung et al., 2002; Hanson et al., 2003; Sasmono et al., 2003; Xue et al., 2003; Fontenot et al., 2005). The numbers of mice used to study cell behavior were: six MMTV-PyMT;ACTB-ECFP;Foxp3\textsuperscript{EGFP}, three MMTV-PyMT;ACTB-ECFP;CD11c-DTR-EGFP, seven MMTV-PyMT;ACTB-ECFP;c-fms-EGFP, three ACTB-ECFP;c-fms-EGFP and two MMTV-PyMT;ACTB-ECFP;Fsp1\textsuperscript{+/EGFP} mice. In addition, four MMTV-PyMT;ACTB-ECFP;Foxp3\textsuperscript{EGFP} mice and three MMTV-PyMT;ACTB-ECFP;c-fms-EGFP mice were used to study cellular responses to acute hypoxia. Two MMTV-PyMT;ACTB-ECFP;c-fms-EGFP mice were injected intravenously with 100 μl of sterile PBS containing 4 mg/ml of 70 kDa rhodamine-conjugated dextran (Invitrogen) and either 75 μg unlabeled and 25 μg Alexa Fluor 647-conjugated anti-Gr1 antibody (clone RB6-8C5, from the UCSF Hybridoma Core), or identical amounts of labeled and unlabeled isotype-matched control antibody. The tumor lesions were divided into broad categories that were recognizable in vivo: hyperplasias were defined as lesions with a diameter of less than 500 μm without excess stromal cell infiltration or invasion fronts; early carcinomas were small to medium lesions with increased stromal cell infiltration and invasion fronts; and late carcinomas were large lesions with compact cancer cells and massive stromal cell infiltration at the stromal border. All animal experiments were conducted in accordance with procedures approved by the Institutional Animal Care and Use Committee, UCSF.

Confocal imaging of live mice

Details of the microscope design and manufacturer information are listed in the supplementary material (Fig. S1). Briefly, a micro-lensed, spinning disk confocal scan-head was coupled to a motorized, inverted fluorescence microscope, and four-color lensed, spinning disk confocal scan-head was coupled to a solid-state laser, and selective emission filters. Images were collected using an ICCD camera (XR-Mega-10EX S-30, Stanford Photonics, Palo Alto, CA), with photon amplification of 20,000× prior to the CCD, whereas other cameras amplify the signal after the CCD. The CCD read-out noise (related to the pixel clock speed) does not limit the photon detection level. The combination of micro-lensed, spinning disk technology, with the ICCD camera technology facilitates the low light imaging that enables the long duration of experiments. For live, long-term, multi-position imaging using a robotic stage, an air lens was selected for ease of use. We obtained the best performance with a 10× Fluar, 0.5 NA lens, which was bright, resulted in negligible photobleaching when using fluorescent proteins, and could provide images from up to 70 μm into the tissue. Furthermore, it collected data from a large region (676 × 676 μm) at 0.66 μm/pixel.

Surgical procedures and anesthesia

Mice were initially anesthetized with 4% isoflurane (at 21% oxygen, balance nitrogen, Summit Anesthesia Solutions, OR), and surgery was performed with 2.5% isoflurane. The ventral surface of the mouse was prepared for surgery with isopropyl alcohol, a ventral midline incision was then made with sterilized scissors, the inguinal mammary fat pad was surgically exposed and a glass microscope slide was glued to the skin behind the mammary gland. The glass slide was rotated to expose the inner surface of the mammary gland, and the mouse was transferred to the microscopic stage. During imaging, isoflurane was reduced to the lowest concentration at which the mouse did not react to pain, typically 0.9-1.1%. We used an oximeter probe (MouseOx, Starr Life Sciences, PA) to monitor and display the heart rate (beats per minute; bpm), the arterial oxygen saturation of the blood (%), and the distension of blood vessels caused by the pulse and breathing (μm). This real-time feedback allowed us to adjust the anesthesia levels to the individual mouse. During the imaging procedure, isoflurane was delivered in a humidified mix of nitrogen and oxygen (at least 21%), with oxygen adjusted to achieve >95% oxygen saturation of arterial blood as measured with an oximeter probe. Mice received 50-100 μl/hour of saline intraperitonally and were covered with a recirculating heated water blanket during imaging (Gaymar, Orchard Park, NY). We have imaged 69 mice of various genotypes with a median time under anesthesia of 7 hours 29 minutes (range: 2 hours 45 minutes-27 hours 29 minutes).

Induction of acute systemic hypoxia

Acute systemic hypoxia was induced by lowering inhaled oxygen concentration to 7% for 20 minutes, followed by normalization with 21% oxygen. During imaging, mice received saline intraperitonally and were covered with a heated blanket.

Histology and immunostaining

Tissue sections (4% paraformaldehyde fixed, paraffin embedded, 5 μm sections) were stained with hematoxylin and eosin using standard methods. For labeling of leukocytes, frozen sections [methanol/acetonitrile (1:1) fixed, 23 μm optical sections] were incubated with FITC-conjugated anti-CD4, or isotype control antibodies (BD Pharmingen) diluted 1:100, washed, and counterstained with 1 μg/ml propidium iodide (Invitrogen). For labeling of CD68 and CD206, frozen sections from MMTV-PyMT;ACTB-ECFP;c-fms-EGFP tumors (harvested 4 hours after intravenous injection of 10 kDa Alexa Fluor 647-conjugated dextran, fixed in 4% paraformaldehyde) were incubated with anti-CD68, anti-CD206 antibody or isotype control antibody (Serotec) diluted 1:50, washed, and then incubated with secondary Alexa Fluor 568-conjugated anti-rat antibody (Invitrogen) diluted 1:150. Staining for CD68 and CD206 was conducted on five tumors from four mice.

FACS analysis of tumor infiltrating leukocytes

Mice were anesthetized with 2.5% avertin, and cardiac perfused with PBS at 110-120 mmHg until the flow-through fluid was clear. Tumors (diameter 8-12 mm) were isolated, chopped with razor blades, and digested with 2 μg/ml collagenase type IV (Sigma C5138) in DME H-21 containing 5% fetal calf serum (FCS) and 5 μg/ml insulin for 30 minutes at 37°C, and then washed with DME H-21 with 5% FCS. The cells were resuspended in DME H-21.
containing 5% FCS and 50 units/ml of DNAse (Sigma D4263), and the suspension slurry was run sequentially through 70 μm and 40 μm meshes. The resulting single cell suspensions were counted, and 5×10^5 cells were incubated with Fc-Block and then with fluorescently conjugated antibodies against cell-type specific markers (BD Biosciences and AbD Serotec) at 4°C for 30 minutes, the cells were then washed twice with PBS (without calcium or magnesium) with 1% FCS. The vital stain 7-ADD was added and the stained populations were analyzed using Becton-Dickinson FACScan and FlowJo software. For characterization of the different leukocyte populations, results from four MMTV-PyMT mice, were analyzed separately, were averaged. For characterization of the cell populations that express c-fms-EGFP in tumors, cells from four MMTV-PyMT;c-fms-EGFP tumors were pooled and analyzed together.

Image analysis and cell tracking
All data sets were corrected for respiratory motion artifacts using Bitplane Imaris (version 5.5 or 5.7 for Windows X64) ‘drift correction’ with spot detection at a minimum diameter of 20 μm and Brownian movement algorithm with a maximum radius of 50 μm. Migration of Tregs and myeloid cells was analyzed using Bitplane Imaris (version 5.7 for Windows X64). Migration of Tregs was tracked manually using the measurement point function of Bitplane Imaris (version 5.7 for Windows X64). All cells present for five or more time points were tracked in six fields from three mice (n=130). Non-migration was defined as an average track length/time of <1 μm/minute and a total displacement over the entire observation period of <10 μm. Cells were considered to have arrested when they stayed within a 10 μm radius for 5 minutes or longer. The distance of migratory Tregs from blood vessels was estimated on 13 fields from 5 mice (n=98) and the median and range of these estimations are reported. Tracking of Tregs before, during, and after acute hypoxia was carried out using ImageJ 1.34 software. Owing to the large number of myeloid cells, their migration was analyzed automatically using Bitplane Imaris with spot detection at a minimum diameter of 8 μm and Brownian movement algorithm with a maximum radius of 14 μm/minute, in 1 hour image sets, with 40-48 seconds between frames, in 200 × 200 μm fields, which more accurately covered one microenvironment than the entire image fields. Velocity, defined as the magnitude of the average velocity vector, was calculated as the ratio between total displacement and total time tracked, and reported for all tracks with duration of at least five time points. For each tissue microenvironment, four or more different fields in at least three different mice were analyzed. For tracking of myeloid cells before, during, and after induction of acute systemic hypoxia, the entire image field was tracked. Cell tracking is intrinsically difficult in live tissues because cells change intensity or diameter as they squeeze through the tissue, and because of limited contrast between individual cells in areas with high cell infiltration. Displacement is, therefore, a more robust measure, and better for comparing single cell migration between mice than track length. Displacement is not sensitive to the time intervals between image acquisition, whereas motion artifacts lead to overestimation of track length with a large mouse-to-mouse variation (Shakhar et al., 2005). However, since cells rarely migrate in straight lines, migratory displacement is shorter than the path length, and the magnitude of the average velocity vector, which we used to compare migration, is much smaller than the maximal instantaneous speed the myeloid cells can achieve in vivo. These challenges are not intrinsic to our technique.

Statistical methods
The distributions of myeloid cells into slow, medium and fast migratory cells in different microenvironments were analyzed by chi-square test using the total number of cells in each category. GraphPad Prism 4 software and alpha=0.05 were used.

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COMPETING INTERESTS
G.P. has a personal financial interest in Solamere Technology Group that custom builds microscopes similar to the one presented in the manuscript and therefore declares competing financial interest.

AUTHOR CONTRIBUTIONS
M.E. and A.J.E. contributed equally to this study. M.E., A.J.E., B.E.W. and Z.W. conceived the imaging strategies; A.J.E. and G.P. designed and built the spinning disk confocal microscope with contributions from M.E. and Z.W.; M.E. and Z.W. designed the experiments with contributions from A.J.E. and M.F.K.; M.E. and H.A.A. did the live imaging experiments; and M.E. and A.J.E. did analysis of live imaging experiments with contributions from A.J.E., M.E., H.A.A. and A.J.E. did immunostainings; M.L.T. did the FACS experiments and the analysis; M.E., A.J.E. and Z.W. wrote the manuscript.

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at dmm.biologists.org/cgi/content/full/1/?/?/DC1

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REFERENCES


