Published in final edited form as:

ACS Chem Biol. 2018 August 17; 13(8): 2339–2346. doi:10.1021/acscchembio.8b00509.

Surface-Modified Macrophages Facilitate Tracking of Breast Cancer-Immune Interactions

Bishnu P. Joshi[#], Joseph Hardie[#], Michael A. Mingroni[#], and Michelle E. Farkas^{*}

Department of Chemistry, University of Massachusetts, 710 North Pleasant Street, Amherst, Massachusetts 01003, United States

[#] These authors contributed equally to this work.

Graphical Abstract

Abstract

The immune system has been found to play key roles in cancer development and progression. Macrophages are typically considered to be pro-inflammatory cells but can also facilitate prooncogenic activities via associations with tumors and metastases. The study of macrophages and their interactions within the context of cancer microenvironments is stymied by the lack of a system to track them. We present a cell-based strategy for studying cancer-immune cell interactions by chemically modifying the surfaces of macrophages with fluorophores. Two widely used methods are employed, affecting cell surface proteins and glycans via NHS-ester and Staudinger ligation reactions, respectively. We show that these modifications do not interfere with macrophage responses to chemoattractants and that interactions with cancer cells can be readily

^{*}Corresponding Author: farkas@chem.umass.edu.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acscchembio.8b00509](https://doi.org/10.1021/acscchembio.8b00509).

Video 1 (AVI)

Video 2 (AVI)

Video 3 (AVI)

Video 4 (AVI)

Video 5 (AVI)

Video 6 (AVI)

Video 7 (AVI)

Video 8 (AVI)

Additional experimental details and figures (PDF)

Notes

The authors declare no competing financial interest.

monitored. This work describes the development of macrophage-based imaging agents for tumor detection and assessment of interactions between immune cells and cancers.

With implications toward the development of new treatments and understanding the nature of the disease, the intersection between cancer and the immune system has become a very busy place. While the immune system is responsible for detecting and removing abnormal cells, many cancers can produce signals and/or undergo transformations to avoid this fate.¹ Macrophages are immune cells that play a major role in facilitating cancer progression (Figure 1), leading to the correlation of their presence with disease severity in many cancer types.² In fact, macrophages represent the most abundant leukocyte within the tumor environment, comprising in some instances up to 50% of the tumor mass.³ Tumor-associated macrophages (TAMs) have been shown to generate factors that promote tumor angiogenesis,⁴ silence the immune response to tumors,² and contribute to the epithelial to mesenchymal transition⁵ (EMT), a metastatic process where epithelial cells undergo changes that result in an enhanced migratory capability, increased invasiveness, and elevated resistance to apoptosis ascribed to mesenchymal (i.e., stem cell-like) phenotypes via remodeling of the tumor environment and association with tumor cells.⁶ They have also been implicated in the metastasis-enabling processes of intra- and extra-vascularization of migratory tumor cells⁷ and can affect the efficacy of anticancer therapeutics.⁸ TAMs are not only important in the initial stages of metastasis but have been shown to contribute to the establishment and survival of metastases at sites away from the primary tumor.^{9–11} TAMs have been associated with a variety of tumor types, including breast, prostate, glioma, lymphoma, bladder, lung, cervical, and melanoma.¹²

On account of their roles in cancer progression and metastasis, TAMs have become a target of interest for developing new treatments.¹³ But because macrophages and their monocyte precursors are actively recruited to cancerous tissues,¹⁴ they have also become candidates for use as imaging and therapeutic agent delivery vehicles.¹⁵ A number of tumor types secrete the major macrophage chemoattractants macrophage colony stimulating factor (M-CSF, also known as colony stimulating factor 1, or CSF-1)¹⁶ and monocyte chemoattractant protein like chemokine (C–C motif) ligand 2 (CCL2).¹⁷ Once in the tumor microenvironment, TAMs can traffic into difficult-to-reach hypoxic regions,¹⁴ areas that are problematic to target with other therapeutic delivery systems.¹⁸ Engineered macrophages are therefore being developed as tools for the diagnosis and treatment of various diseases, from delivering theranostic (therapeutic and diagnostic) agents to tumors¹⁹ to administering antiretroviral therapeutics to HIV-infected mice.²⁰ For cancer-based applications, the specific interactions of macrophages with tumor tissue have been exploited to facilitate imaging of tumors and metastases and the delivery of various therapeutics. This has enabled enhanced contrast of tumor boundaries and imaging of metastases, as well as the delivery of nanoparticle-conjugated small molecule and photothermal therapeutics to tumors, showing efficacy *in vitro* and *in vivo*.^{19,21–24}

The challenge in using nanoparticles as a therapeutic strategy, however, is that their phagocytosis and release are difficult to control, despite modifications to particle surfaces to alter their characteristics.²⁵ Furthermore, because small molecules cannot be engulfed and

released in the same manner without additional modifications,²⁶ these platforms are limited to the use of nanoparticle agents. Separately, transgenic animals expressing GFP,²⁷ CFP,²⁸ and RFP²⁹ have been produced and employed in imaging studies of cancer–host cell interactions,³⁰ including in longitudinal studies.³¹ In these cases, tumor cells derived from a fluorescent animal can be distinguished from host cells in or from another animal bearing a different reporter.^{32,33} While cells derived from these systems can be used in the context of imaging, they must be obtained from genetically modified animals, and only fluorescence-detecting platforms may be used. To circumvent various issues associated with macrophage engulfment and release, and to provide a more flexible strategy for imaging, delivery, and studies of macrophage associations with cancer, we have investigated the direct modification of macrophage cell surfaces with small molecules.

The cell membrane contains a diverse array of biomolecules, many of which can be chemically manipulated³⁴ to allow selective noncovalent³⁵ and covalent bioconjugations.³⁶ Successful membrane modification involves linkage of the target molecule to the cell surface under physiological conditions, without inhibiting the normal functioning of the cell. In this report, we demonstrate that fluorescent probes can be appended to macrophages to monitor chemosensing, tracking, and interactions with cancer cells. Using either *N*-hydroxysuccinimide coupling chemistry³⁷ or metabolic incorporation of unnatural azido-sugars,³⁸ we show that modified macrophages chemotax to a similar extent as unmodified cells and, more importantly, continue to associate with cancer cells *in vitro* and accumulate in tumors *in vivo*. This work sets the stage for further use of this platform as a diagnostic tool but also as a delivery agent for therapeutics and molecular probes to study the tumor microenvironment.

RESULTS AND DISCUSSION

Modification of Macrophages to Install Fluorescent Molecules.

To determine whether macrophages (i.e., RAW 264.7 cells) are amenable to surface modifications, three different approaches were used (Figure 2A, Supporting Information Table S1 and Figure S1). In the first method, the cell surface is biotinylated by reacting exposed primary amines with sulfosuccinimidyl-6(biotinamido)hexanoate (Sulfo-NHS-LC-Biotin), prior to binding fluorescein isothiocyanate (FITC)-labeled avidin.³⁹ Cellular labeling was confirmed by fluorescent confocal microscopy (Figure 2B, Supporting Information Figure S2A). We also attached the dye moiety directly to the cells, forgoing the display of large avidin proteins on the cell surface, via *N*-hydroxysuccinimide (NHS)-dye conjugation. With Sulfo-NHS-Cyanine5 (NHSCy5), we observed significant cellular modification (Figure 2C, Supporting Information Figure S2B). As a third approach, we used metabolic labeling and “click” chemistry, which also utilizes small molecules, but modifies incorporated unnatural azido-glycans as opposed to amino acids.³⁸ Cells were metabolically labeled with azidoacetylmannosamine (ManNAz) or azidoacetylglucosamine (GlcNAz), and subsequent Staudinger ligation was performed using Phosphine-Dylight 650 (Phos-Dy680). Confocal microscopy images illustrate the extent of labeling (Figure 2D, Supporting Information Figure S2C).

All three conjugation approaches resulted in significant cellular modification with a majority of the dye intensities located at the cell membranes. As expected, fluorescence intensity decreases concomitantly with macrophage proliferation (doubling time is ~15 h; Supporting Information Figure S3) and the signal remains over several cell divisions. Furthermore, these treatments largely resulted in no apparent cellular toxicity (Supporting Information Figure S4). Following modifications, cells could be stored and were viable for up to 1 week without any manipulation, with the exception of metabolically installed ManNAz conjugates, which showed slightly diminished viabilities (data not shown). With the two-step strategies (biotin-avidin and metabolic labeling), we have found that the appendage of the fluorophores largely depends on the presence of the linker. For example, incorporation of avidin-FITC is not observed unless cells have been biotinylated (Figure 3A). Likewise, minimal uptake of Phos-Dy680 is detected in cells that have not incorporated the azido sugar (Figure 3B). For the biotin/avidin-dye conjugation, the extent of cellular labeling with various avidin-dye concentrations and incubation times was determined (Supporting Information Figure S5); hundreds of millions to billions of dye molecules could be incorporated. Modifications did not affect macrophage polarization state (Supporting Information Figure S6), and cells largely retained their abilities to phagocytose entities (Supporting Information Figure S7).

The employment of these three strategies is also useful for the modification of cells with other molecules; a wide array of NHS-, (strept)avidin-, and phosphine-linked molecules are commercially available or can be synthesized. Of particular interest for *in vivo* imaging applications is the installation of near-infrared dyes to facilitate tissue penetration (Supporting Information Figure S8). We have also found the surface functionalization methods to be versatile and amenable to use with other macrophage types, including J774.2 (murine monocyte macrophages) and bone marrow derived primary macrophages (Figure 3C).

Macrophage Migration and Chemotaxis.

Critical to the employment of functionalized macrophages as agents for the visualization of macrophage interactions with cancer cells, delivery of therapeutics to tumor sites, or localization of chemical probes to understand oncogenic microenvironments, is the retention of chemotactic properties. For this reason, it is important to assess motility following chemical modification. Because the biotin/avidin-dye modification results in the potential for steric hindrance on account of the resulting display of avidin proteins (monomer is approximately 16.5 kDa), the majority of studies described here were conducted using macrophages that were conjugated in this manner.

Wound healing/scratch assays were used to visualize cellular motility.⁴⁰ A single “scratch” was generated through a monolayer of cells, and the ability of the cells to migrate and fill the scratch was tracked over time via fluorescence microscopy. We compared nonmodified cells with those appended with biotin/avidin-FITC (Figure 4A) and noted that the two were strikingly similar. Macrophage response to chemotactic signals was determined via Boyden chamber assay.⁴¹ Here, labeled and nonlabeled macrophages were compared in their abilities to migrate through a membrane in response to a chemoattractant (CSF-1 was used in all instances; Figure 4B, Supporting Information Figure S9A). These experiments show that not

only do cells survive chemical modification but they continue to migrate toward a chemoattractant. Furthermore, even the presence of large avidin proteins on the cell surface does not hinder cellular sensing and trafficking abilities.

Having determined that the biotin/avidin-dye modification method, which results in the largest change to the cell surface, has minimal effects on cellular chemotaxis, we evaluated the effects of the other labeling strategies. Boyden chamber assays were also used to determine the effects of direct NHS-dye incorporation and metabolic-Staudinger ligation methods (using both GlcNaz and ManNaz for azide incorporation) on migration toward CSF-1 chemoattractant (Figure 5, Supporting Information Figure S9B). Similarly to the biotin/avidin-FITC incorporation, the use of these other strategies resulted in minimal change to migration ability in comparison with nonmodified cells exposed to CSF-1 in the positive control group. As a result, we conclude that each method assessed largely preserves the ability of macrophages to track and follow chemoattractant signals produced by cancer cells and may be amenable to use in further studies on macrophage-oncogenic interactions.

Macrophage Association with *in Vitro* Models of Breast Cancer.

After establishing that the migratory aptitude of macrophages is not altered upon modification, we investigated the association between these modified cell lines and cancer cells. Breast cancer is a heterogeneous disease generally classified into five subtypes based on genetic profile: luminal A (estrogen receptor (ER) positive, low grade), luminal B (ER positive, high grade), human epidermal growth factor receptor 2 (HER2) enriched, basal-like (ER negative, HER2 negative, progesterone receptor (PR) negative; often referred to as triple negative), and claudin-low (triple negative with low expression of cell–cell junction proteins).⁴² These disease types are not only associated with the presence and/or absence of cellular markers but with varying levels of aggression and patient outcomes. At different ends of the spectrum, luminal A is generally considered highly treatable and has high rates of survival, while triple negative types are extremely aggressive with few treatments available, resulting in far worse prognoses. Knowing that macrophages are strong contributors to cancer progression,^{5,10} that patients with higher levels of tumor associated macrophages have worse prognoses,³ and that CSF-1 both is a macrophage chemoattractant and has been correlated with breast cancer mortality,⁴³ we wished to determine whether particular cancer subtypes have an enhanced capability to recruit and interact with macrophages.

We present here the results from initial studies addressing the association of macrophages with cancer cells representing different subtypes and degrees of aggression, facilitated by chemically modified macrophages. While it has been observed that macrophage infiltration occurs to a lesser extent in luminal A versus other tumor subtypes,⁴⁴ interactions between cancer cells and macrophages have not been assessed. We utilize MCF7 (luminal A), SKBR3 (HER2+), and MDA-MB-231 (triple negative, claudin-low) cell lines in concert with RAW 264.7 macrophages at approximately a 2:1 ratio. Previous work has demonstrated the cross-species interaction of murine macrophages with human cancer cell lines.⁴⁵ Indeed, across multiple experiments the macrophages show the most vivid associations with the most aggressive cell line, MDA-MB-231 (Figure 6 and Supporting Information Video 1).

Fluorescence microscopy was similarly used to evaluate macrophage interactions with MCF7 and SKBR3 cells (Supporting Information Figure 10 and Videos 2 and 3). While macrophages associated with both of these breast cancer cell lines as well, the interactions were not nearly as dramatic. Whereas the macrophages appear to “pick up” and move the MDA-MB-231 cells, they seem to pull the MCF7 cells, which remain attached to the surface. The SKBR3 cells appear unperturbed by the macrophages, which hover in the vicinity and appear to make contact, but do not show any effect. Single-cell type experiments are provided for reference (Supporting Information Videos 4–8). Future work toward this end involves studying associations of macrophages with additional cell types and the use of three-dimensional cell culture tissue models.

Chemically Modified Macrophages Show Tumor Homing Capabilities in a Mouse Model of Cancer.

In vivo biodistribution studies of functionalized macrophages were performed to determine the applicability of our platform to studying the interactions between macrophages and tumors and metastases in mouse models of cancer. Because of the immune-relevant nature of this work, it is critical to use animals with intact immune systems. For this reason, we used female BALB/c mice orthotopically implanted with isogenic 4T1 (mouse mammary carcinoma) cells;⁴⁶ RAW 264.7 macrophage cells also possess the same genetic background. 4T1 cells are highly tumorigenic and invasive. They have been widely used as a clinically relevant triple-negative breast cancer model and are considered to represent stage IV human breast cancer.⁴⁷ Once tumors were palpable, macrophage cells were modified using the biotin/avidin-dye method to append Dy680 to the cell surface. These cells were intravenously injected into the mice ($n = 3$) via tail veins. Because the mouse breed possesses autofluorescent hair, the removal of which can result in additional stress and inflammation and is not always effective in removing all signal, macrophage biodistribution was assessed *ex vivo* following euthanasia at 4 and 24 h following injection (Figure 7, Supporting Information Figure S11).

At the 4 h time-point, significant signal is observed in the liver, followed by the spleen, lungs, and tumor, with some signal in the brain. It is also noted that significant signal appears in the tail, which may be the result of macrophage accumulation near the site of injection due to the presence of a wound or inability to leave the tail vein. After 24 h, signal remains in the tumor, liver, and spleen. Considering that the macrophage surfaces are significantly modified with foreign entities (Figure 2), the accumulation of macrophages in the liver is not surprising. The hepatic route is a major pathway for elimination of a variety of drugs, nanoparticles, and other entities that are too large for renal (kidney) excretion. It is in fact encouraging that in general, macrophages that are not localized to the tumor are also not accumulating in other tissues but rather are likely being excreted. In the future, we will assess the biodistribution of macrophages modified with smaller functionalities. Also, in further work, we seek to use imaging modalities that will facilitate *in vivo* tracking of the macrophages (e.g., PET or MRI) and study their tracking to and accumulation at metastatic sites.

CONCLUSION

We have been able to demonstrate that macrophages can be functionalized via three different approaches, retaining viability and their inherent migratory and chemotactic properties *in vitro* and *in vivo*. While earlier work utilizing macrophages as delivery vehicles was constrained to the phagocytosis and release of nanoparticle-based agents, we have shown that surface modification is also feasible, and small molecules may be used. By using this strategy we can now employ macrophage conjugates as delivery vehicles for *in vivo* imaging, therapeutic, and chemical-sensing agents for the diagnosis, treatment, and study of cancer. Furthermore, many groups are broadly interested in the chemical modification of cells toward a variety of applications—this work also serves to answer some fundamental questions regarding the biological effects of these alterations.

METHODS

Reagents and Cell Lines.

All reagents were purchased from Thermo-Fisher Scientific except where otherwise noted. Immortalized cell lines were obtained from the ATCC and maintained under ATCC-recommended conditions. Primary macrophages were isolated and differentiated from bone marrow of BALB/c mice, as previously reported.⁴⁸ Following differentiation, cells were used within 7 days.

Biotin-(Strept)avidin Modification of Macrophages.

Cells were labeled in either an adherent or suspended manner. For adherent labeling, culture medium was removed and cells rinsed twice with phosphate-buffered saline (PBS). Cells were then incubated in 2 mM Sulfo-NHS-LC-Biotin for 30 min at ambient temperature and then washed twice with 100 mM glycine, once with PBS, and then incubated in 2.5 $\mu\text{g/mL}$ Avidin-FITC or Streptavidin-Dylight for 30 min at 37 °C/5% CO₂. Cells were rinsed once more with PBS before use. For suspended labeling, cells were harvested via trypsinization, centrifugation, and resuspension, followed by counting. For 6×10^6 cells, the cell pellet was rinsed twice with 1 mL of PBS, centrifuging and removing supernatant for each wash. Cells were resuspended in 2 mL of 2 mM Sulfo-NHS-LC-Biotin and incubated for 30 min at ambient temperature. Cells were then centrifuged at 1500 rpm for 5 min, and the pellet was washed twice with 2 mL of 100 mM glycine and once with 1 mL of PBS. Cells were resuspended in 2 mL of 2.5 $\mu\text{g/mL}$ FITC-Avidin or Streptavidin-Dylight 680 for 30 min at 37 °C and 5% CO₂. Cells were centrifuged at 1500 rpm for 5 min, and the pellet was rinsed once with 1 mL of PBS before use. Images of cells were acquired using a Nikon Point Scanning C2+ confocal microscope with excitation at 488 and 650 nm.

Direct NHS-Ester Modification of Macrophages.

Cells were labeled in either an adherent or suspended manner. For adherent labeling, culture medium was removed and cells rinsed twice with phosphate-buffered saline (PBS). Cells were then incubated in 100 μM Sulfo-NHS-Cyanine5 (Lumiprobe) for 1 h at 37 °C and 5% CO₂, and then washed twice with 100 mM glycine, once with PBS. Cells were rinsed once more with PBS before use. For suspended labeling, the cell monolayer was treated with

0.25% trypsin for detachment, centrifuged, resuspended, and counted. For 4×10^6 cells, the pellet was rinsed twice with 1 mL of PBS, centrifuging and removing supernatant for each wash. Then, cells were suspended in 400 μL of 100 μM Sulfo-NHS-Cyanine5 (Lumiprobe) and incubated for 1 h at 37 °C and 5% CO_2 . Cells were centrifuged at 1500 rpm for 5 min, and the pellet was rinsed twice with 2 mL of 100 mM glycine and once with 1 mL of PBS before use. Images of cells were acquired using a Nikon Point Scanning C2+ confocal microscope with excitation at 488 and 650 nm.

Metabolic Labeling/Staudinger Ligation Modification of Macrophages.

Labeling of cells via this method largely followed previously established protocols.²⁸ In both suspended and adherent modifications, cells were cultured in complete DMEM media supplemented with 40 μM ManNAz or GlcNAz (0.4% DMSO v/v), for 72 h at 37 °C and 5% CO_2 . For adherent labeling, culture medium was removed and cells rinsed twice with phosphate-buffered saline (PBS). Cells were then incubated in Dylight 650-Phosphine (1% DMSO v/v) and incubated for 3 h at 37 °C and 5% CO_2 and then washed twice with PBS. Cells were rinsed once more with PBS before use. For suspended modification, cells were detached using a cell scraper. For 4×10^6 cells, following additional centrifugation, the pellet was rinsed with 1 mL of 2% fetal bovine serum (FBS) in Hank's Buffered Saline Solution (HBSS), and then resuspended in 400 μL of 100 μM Dylight 650-Phosphine (1% DMSO v/v) and incubated for 3 h at 37 °C and 5% CO_2 . Cells were centrifuged at 1500 rpm for 5 min, and the pellet was rinsed twice with 1 mL of 2% FBS in HBSS and once with 1 mL of PBS before use. Images of cells were acquired using a Nikon Point Scanning C2+ confocal microscope with excitation at 488 and 650 nm. For experiments employing a DMSO control, populations of cells were treated with DMSO for analogous times at the same concentrations (0.4% for 72 h and 1% for 3 h).

Wound Healing/Scratch Assay.

For the wound healing assay, RAW 264.7 cells were plated at high density (5×10^6 cells per well) in a six-well plate and incubated for 6 h at 37 °C and 5% CO_2 to adhere. The cells were labeled with avidin-FITC according to the protocol above. A sterile 200 μL pipet tip was used to make a single scratch through the monolayer. The cells were rinsed once with PBS and then incubated in phenol-red free complete DMEM media. Scratch width was monitored using a Zeiss Axio Observer Z1 microscope.

Chemotaxis/Boyden Chamber Assay.

Boyden chamber cell migration assays were largely performed as previously described.³¹ A transwell insert (8 μm pore, 6.5 mm, PET membrane; Corning Life Sciences) was coated with 10 $\mu\text{g}/\text{mL}$ of fibronectin (Sigma-Aldrich), allowed to rest for 4 h at ambient temperature, rinsed with PBS, and left to dry overnight. The inserts were placed into 24-well plates containing 650 μL of starved (FBS-free) media supplemented with and without 40 ng/mL rCSF-1, depending on chemoattractant conditions. In parallel, cells were cultured for 24 h in starved media and then either labeled according to the respective protocol or left untreated. For azido-sugar metabolic labeling, cells were cultured in complete media containing the azido-sugar for 48 h, followed by replacement with starved media containing azido-sugar for 24 h. For each cell sample, 100 μL containing 1×10^5 cells was added into

the transwell insert and incubated for 12 h at 37 °C and 5% CO₂. Nonmigratory cells were removed with a Q-tip, and migratory cells at the bottom of the insert were fixed in 4% formaldehyde and stained with a 0.1% crystal violet solution in 25% methanol. Membranes were removed from the inset, mounted onto cover-glass, and visualized using a Zeiss Axio Observer Z1 with an Axio Cam 506 Color attachment. Using a 20× objective, cells were counted from three fields of view per membrane, with three membranes per condition ($n = 9$). Box and whisker plots were generated using OriginPro 2017, and statistical significance was determined using the student's t test (two tailed distribution and two sample unequal variance). The presence of fibronectin was determined to not have any significant effect on migration (Supporting Information Figure S12).

Coculture Assays.

MCF7, SKBR3, and MDA-MB-231 cells were plated on 24-well tissue culture plates at 1×10^5 cells per well and incubated overnight (~20 h). MDA-MB-231 cells were labeled with CellTracker Blue CMAC dye (Invitrogen) according to the manufacturer's protocol. RAW 264.7 cells were avidin-FITC labeled in suspension, and 1×10^5 cells were added to each cancer-cell-containing well. Accounting for cancer cell growth, the ratio of cancer to macrophage cells was approximately 2:1. Time lapse microscopy was used to monitor cell behavior at 15 min intervals over 12 h using a Zeiss Spinning Disk Observer SD confocal microscope with excitation at 405 and 488 nm.

Generation of *in Vivo* Tumor Models and Macrophage Biodistribution Studies.

Six to eight-week-old BALB/cAnNCrl mice (Charles River Laboratories) were orthotopically implanted with 5×10^4 4T1 murine breast cancer cells, similarly to previous studies.³⁶ Briefly, mice were anesthetized with 400 mg/kg tribromoethanol, the ventral thoracic-inguinal region was shaved, and an incision was made to expose the fourth mammary fat pad. The veins leading to the fat pad were cauterized and the fat pad was removed using forceps. The 4T1 cells were injected into the fat pad cavity in 10 μ L of PBS using a 100 μ L Hamilton syringe. A total of 1 mg/kg of bupivacaine was administered to the surgical site, and the wound was closed using wound clips. Following closure, 1 mg/kg meloxicam was administered subcutaneously.

Mice to be used for imaging were placed on an alfalfa-free diet. Once palpable tumors formed, 1×10^7 RAW 264.7 cells were labeled with avidin-DY680 using the suspension method described above. Cells were then suspended in 100 μ L of PBS and were injected into tumor-bearing mice intravenously through the tail vein. At 4 and 24 h following injection, mice were euthanized, and organs, blood, and tumors collected. Tissues were imaged using an In Vivo Imaging System (IVIS; PerkinElmer) available at UMass Amherst. All procedures involving the use of animals were conducted under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at UMass Amherst.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors would like to thank S. Peyton and her laboratory for their assistance in performing time-lapse microscopy experiments and generously allowing us to use their microscopes. We also thank A. Burnside and the UMass Amherst animal facility staff for assistance with IVIS *ex vivo* imaging experiments and animal husbandry, respectively.

Funding

This research was supported by a SEED grant from the University of Massachusetts Institute for Applied Life Sciences. J.H. was supported by a National Research Service Award, T32 GM008515.

REFERENCES

- (1). Grivennikov SI, Greten FR, and Karin M (2010) Immunity, inflammation, and cancer. *Cell* 140, 883–899. [PubMed: 20303878]
- (2). Qian B-Z, and Pollard JW (2010) Macrophage diversity enhances tumor progression and metastasis. *Cell* 141, 39–51. [PubMed: 20371344]
- (3). Zhang Y, Cheng S, Zhang M, Zhen L, Pang D, Zhang Q, and Li Z (2013) High-infiltration of tumor associated macrophages predicts unfavorable clinical outcome for node-negative breast cancer. *PLoS One* 8, e76147. [PubMed: 24098773]
- (4). Owen JL, and Mohamadzadeh M (2013) Macrophages and chemokines as mediators of angiogenesis. *Front. Physiol.* 4, 159. [PubMed: 23847541]
- (5). Bonde A-K, Tischler V, Kumar S, Soltermann A, and Schwendener RA (2012) Intratumoral macrophages contribute to epithelial-mesenchymal transition in solid tumors. *BMC Cancer* 12, 35. [PubMed: 22273460]
- (6). Condeelis J, and Pollard JW (2006) Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 124, 263–266. [PubMed: 16439202]
- (7). Wyckoff JB, Wang Y, Lin EY, Li JF, Goswami S, Stanley ER, Segall JE, Pollard JW, and Condeelis J (2007) Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res.* 67, 2649–2656. [PubMed: 17363585]
- (8). Mantovani A, and Allavena P (2015) The interaction of anticancer therapies with tumor-associated macrophages. *J. Exp. Med.* 212, 435. [PubMed: 25753580]
- (9). Su S, Liu Q, Chen J, Chen J, He C, Huang D, Wu W, Lin L, Huang W, Zhang J, Cui X, Zheng F, Li H, Yao H, Su F, Song E, and Chen F (2014) A positive feedback loop between mesenchymal-like cancer cells and macrophages is essential to breast cancer metastasis. *Cancer Cell* 25, 605–620. [PubMed: 24823638]
- (10). Chen Q, Zhang XH, and Massague J (2011) Macrophage binding to receptor VCAM-1 transmits survival signals in breast cancer cells that invade the lungs. *Cancer Cell* 20, 538–549. [PubMed: 22014578]
- (11). Qian B, Deng Y, Im JH, Muschel RJ, Zou Y, Li J, Lang RA, and Pollard JW (2009) A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment, and growth. *PLoS One* 4, e6562. [PubMed: 19668347]
- (12). Komohara Y, Jinushi M, and Takeya M (2014) Clinical significance of macrophage heterogeneity in human malignant tumors. *Cancer Sci.* 105, 1–8. [PubMed: 24168081]
- (13). Ries CH, Runza V, Wartha K, Cannarile MA, Hoves S, Rey-giraud F, Pradel LP, Feuerhake F, Klamann I, Jones T, Jucknischke U, Scheiblich S, Kaluza K, Gorr IH, Walz A, Abiraj K, Cassier PA, Sica A, Gomez-roca C, de Visser KE, Italiano A, Le Tourneau C, Delord J, Levitsky H, Blay J, Ruttinger D, and Benz J (2014) Targeting tumor-associated macrophages with anti-CSF-1R antibody reveals a strategy for cancer therapy. *Cancer Cell* 25, 846–859. [PubMed: 24898549]
- (14). Chaturvedi P, Gilkes DM, Takano N, and Semenza GL (2014) Hypoxia-inducible factor-dependent signaling between triple-negative breast cancer cells and mesenchymal stem cells promotes macrophage recruitment. *Proc. Natl. Acad. Sci. U. S. A.* 111, E2120–9. [PubMed: 24799675]

- (15). Joshi BJ, Hardie J, and Farkas ME (2018) Harnessing biology to deliver therapeutic and imaging entities via cell-based methods. *Chem. - Eur. J*, DOI: 10.1002/chem.201706180.
- (16). Pixley FJ, and Stanley ER (2004) CSF-1 regulation of the wandering macrophage: complexity in action. *Trends Cell Biol.* 14, 628–638. [PubMed: 15519852]
- (17). Qian B-Z, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, Kaiser EA, Snyder LA, and Pollard JW (2011) CCL2 recruits inflammatory monocytes to facilitate breast-tumor metastasis. *Nature* 475, 222–225. [PubMed: 21654748]
- (18). Brown JM, and Wilson WR (2004) Exploiting tumour hypoxia in cancer treatment. *Nat. Rev. Cancer* 4, 437–447. [PubMed: 15170446]
- (19). Choi J, Kim HY, Ju EJ, Jung J, Park J, Chung HK, Lee JS, Lee JS, Park HJ, Song SY, Jeong SY, and Choi EK (2012) Use of macrophages to deliver therapeutic and imaging contrast agents to tumors. *Biomaterials* 33, 4195–4203. [PubMed: 22398206]
- (20). Dou H, Destache CJ, Morehead JR, Mosley RL, Boska MD, Kingsley J, Gorantla S, Poluektova L, Nelson JA, Chaubal M, Werling J, Kipp J, Rabinow BE, and Gendelman HE (2006) Development of a macrophage-based nanoparticle platform for antiretroviral drug delivery. *Blood* 108, 2827–2835. [PubMed: 16809617]
- (21). Li Z, Huang H, Tang S, Li Y, Yu X, Wang H, Li P, Sun Z, Zhang H, Liu C, and Chu PK (2016) Small gold nanorods laden macrophages for enhanced tumor coverage in photothermal therapy. *Biomaterials* 74, 144–154. [PubMed: 26454052]
- (22). Miller MA, Zheng Y, Gadde S, Pfirschke C, Zope H, Engblom C, Kohler RH, Iwamoto Y, Yang KS, Askevold B, Kolishetti N, Pittet M, Lippard SJ, Farokhzad OC, and Weissleder R (2015) Tumour-associated macrophages act as a slow-release reservoir of nano-therapeutic Pt(IV) pro-drug. *Nat. Commun.* 6, 1–13.
- (23). Choi M, Bardhan R, Stanton-maxey KJ, Cao N, Halas NJ, Clare SE, Badve S, Nakshatri H, and Stantz KM (2012) Delivery of nanoparticles to brain metastases of breast cancer using a cellular Trojan horse. *Cancer Nanotechnol.* 3, 47–54. [PubMed: 23205151]
- (24). Choi M-R, Stanton-Maxey KJ, Stanley JK, Levin CS, Bardhan R, Akin D, Badve S, Sturgis J, Robinson JP, Bashir R, Halas NJ, and Clare SE (2007) A cellular Trojan horse for delivery of therapeutic nanoparticles to tumors. *Nano Lett.* 7, 3759–3765. [PubMed: 17979310]
- (25). Weissleder R, Nahrendorf M, and Pittet MJ (2014) Imaging macrophages with nanoparticles. *Nat. Mater.* 13, 125–138. [PubMed: 24452356]
- (26). Dass CR, and Choong PFM (2006) Targeting of small molecule anticancer drugs to the tumour and its vasculature using cationic liposomes: lessons from gene therapy. *Cancer Cell Int.* 6, 17. [PubMed: 16792817]
- (27). Yang M, Reynoso J, Jiang P, Li L, Moossa AR, and Hoffman RM (2004) Transgenic nude mouse with ubiquitous green fluorescent protein expression as a host for human tumors. *Cancer Res.* 64, 8651–8656. [PubMed: 15574773]
- (28). Tran Cao HS, Reynoso J, Yang M, Kimura H, Kaushal S, Snyder CS, Hoffman RM, and Bouvet M (2009) Development of the transgenic cyan fluorescent protein (CFP)-expressing nude mouse for “technicolor” cancer imaging. *J. Cell. Biochem.* 107, 328–334. [PubMed: 19306297]
- (29). Yang M, Reynoso J, Bouvet M, and Hoffman RM (2009) A transgenic red fluorescent protein-expressing nude mouse for color-coded imaging of the tumor microenvironment. *J. Cell. Biochem.* 106, 279–284. [PubMed: 19097136]
- (30). Hoffman RM (2005) The multiple uses of fluorescent proteins to visualize cancer in vivo. *Nat. Rev. Cancer* 5, 796–806. [PubMed: 16195751]
- (31). Suetsugu A, Katz M, Fleming J, Truty M, Thomas R, Saji S, Moriwaki H, Bouvet M, and Hoffman RM (2012) Noninvasive fluorescent-protein imaging of orthotopic pancreatic-cancer-patient tumorgraft progression in nude mice. *Anticancer Res.* 32, 3063–3068. [PubMed: 22843874]
- (32). Yang M, Li L, Jiang P, Moossa AR, Penman S, and Hoffman RM (2003) Dual-color fluorescence imaging distinguishes tumor cells from induced host angiogenic vessels and stromal cells. *Proc. Natl. Acad. Sci. U. S. A.* 100, 14259–14262. [PubMed: 14614130]
- (33). Hoffman RM, and Yang M (2006) Color-coded fluorescence imaging of tumor-host interactions. *Nat. Protoc.* 1, 928–935. [PubMed: 17406326]

- (34). Kellam B, De Bank PA, and Shakesheff KM (2003) Chemical modification of mammalian cell surfaces. *Chem. Soc. Rev.* 32, 327–337. [PubMed: 14671788]
- (35). Wilson JT, Krishnamurthy VR, Cui W, Qu Z, and Chaikof EL (2009) Non-covalent cell surface engineering with cationic graft copolymers. *J. Am. Chem. Soc.* 131, 18228. [PubMed: 19961173]
- (36). Cheng H, Kastrup CJ, Ramanathan R, Siegwart DJ, Ma M, Bogatyrev SR, Xu Q, Whitehead KA, Langer R, and Anderson DG (2010) Nanoparticulate cellular patches for cell-mediated tumortropic delivery. *ACS Nano* 4, 625–631. [PubMed: 20121215]
- (37). Koniev O, and Wagner A (2015) Developments and recent advances in the field of endogenous amino acid selective bond forming reactions for bioconjugation. *Chem. Soc. Rev.* 44, 5495–5551. [PubMed: 26000775]
- (38). Laughlin ST, and Bertozzi CR (2007) Metabolic labeling of glycans with azido sugars and subsequent glycan-profiling and visualization via Staudinger ligation. *Nat. Protoc.* 2, 2930–2944. [PubMed: 18007630]
- (39). Livnah O, Bayer EA, Wilchek M, and Sussman JL (1993) Three-dimensional structures of avidin and the avidin-biotin complex. *Proc. Natl. Acad. Sci. U. S. A.* 90, 5076–5080. [PubMed: 8506353]
- (40). Rodriguez LG, Wu X, and Guan JL (2004) Wound-healing assay. *Cell Migration* 294, 23–29.
- (41). Chen HC (2004) Boyden chamber assay. *Cell Migration* 294, 15–22.
- (42). The Cancer Genome Atlas Network. (2012) Comprehensive Molecular Portraits of Human Breast Tumors. *Nature* 490, 61–70. [PubMed: 23000897]
- (43). Richardsen E, Uglehus RD, Johnsen SH, and Busund L-T (2015) Macrophage-colony stimulating factor (CSF1) predicts breast cancer progression and mortality. *Anticancer Res.* 35, 865–874. [PubMed: 25667468]
- (44). Gwak JM, Jang MH, Kim DI, Seo AN, and Park SY (2015) Prognostic value of tumor-associated macrophages according to histologic locations and hormone receptor status in breast cancer. *PLoS One* 10, e0125728. [PubMed: 25884955]
- (45). Sousa S, Brion R, Lintunen M, Kronqvist P, Sandholm J, Monkkonen J, Kellokumpu-Lehtinen P-L, Lanttia S, Tynnenen O, Joensuu H, Heymann D, and Maatta JA (2015) Human breast cancer cells educate macrophages toward the M2 activation status. *Breast Cancer Res.* 17, 101. [PubMed: 26243145]
- (46). Pulaski BA, and Ostrand-Rosenberg S (2001) Mouse 4T1 breast tumor model. *Curr. Protoc. Immunol.* 20 (20), 2. [PubMed: 18432775]
- (47). Gregorio AC, Fonseca NA, Moura V, Lacerda M, Figueiredo P, Simoes S, Dias S, and Moreira JN (2012) Inoculated cell density as a determinant factor of the growth dynamics and metastatic efficiency of a breast cancer murine model, *PLoS One*. *PLoS One* 11, e0165817.
- (48). Weischenfeldt J, and Porse B (2008) Bone marrow-derived macrophages (BMM): isolation and applications. *Cold Spring Harb. Protoc.* 12, prot5080.

Figure 1.
Macrophage contributions to cancer and its metastasis.

Figure 2.

Approaches for chemical modification of macrophages. (A) Three methods used to modify the model macrophage cell line RAW 264.7 are an attachment of NHS-biotin followed by noncovalent interaction with fluorophore-conjugated avidin (Biotin/Avidin-dye), amide formation through direct linkage of NHS-fluorophores with cell surface lysines (NHS-dye), and bioorthogonal Staudinger ligation between phosphine conjugates and metabolically incorporated azido sugars (N₃/Phos-dye). Confocal microscopy images show suspended RAW 264.7 cells labeled with (B) avidin-FITC, (C) NHS-Cy5, and (D) phosphine-Dylight

650. Corresponding cellular dye distributions via fluorescence intensities are shown adjacent to the respective image. Modified sites are generally located at the membrane or endosomally throughout the cell (yellow line). Magnification = 60 $\times$, scale bar = 25 μm .

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3.

Specific cellular labeling in the presence of both linkers and dyes, which may be applied to different macrophage types. (A) Suspended cells are only labeled in the presence of both biotin and avidin; no fluorescence is observed where cells are incubated with only avidin. (B) Significant fluorescence is observed in suspended cells metabolically labeled with azidomannose followed by reaction with Dylight 650; in the absence of azidomannose, minimal fluorescence was seen. Magnification = 60 $\times$, scale bar = 25 μ m. (C) The biotin–avidin strategy is used to label J774.2 (monocyte-derived, magnification = 63 $\times$, scale bar =

20 μm) and primary (harvested and differentiated from bone marrow, magnification = 20 $\times$, scale bar = 50 μm) macrophages in suspension.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4.

Motility and chemotaxis capabilities retained by functionalized macrophages. (A) Assessment of adherent macrophage motility and proliferation via wound healing/scratch assay. At time 0, a pipettip-induced scratch was generated in both nonmodified and Avidin-FITC labeled RAW 264.7 cells. Ability of the cells to migrate and proliferate across the scratch after 24 h was observed; both sets of cells behaved similarly. Dashed line indicates border of cells at highest density; scale bar is 200 μm . (B) Boyden chamber assay was used to determine changes in migration characteristics following chemical modification using suspended methods. Modified RAW 264.7 cells migrate similarly to nontreated cells. Nonmodified RAW264.7 cells were tracked in starved media with and without the chemoattractant Colony-Stimulating Factor-1 (CSF) and compared to Avidin-FITC modified cells exposed to CSF. Nine panels of cells were counted per treatment ($n = 9$, from three

biological replicates, represented by differently colored diamonds). Boxes represent the interquartile range (25th to 75th percentile). The line bisecting the box represents the median. The small square in the center is the mean, and whiskers indicate the 5th and 95th percentiles. * $P < 0.0001$.

Figure 5.

Similar behavior of modified suspended macrophages to one another and nonlabeled cells exposed to CSF-1. Box and whisker plot of counted cell groups ($n = 9$ from three biological replicates, represented by differently colored diamonds) from Boyden chamber assay comparing migratory behaviors. Boxes represent the interquartile range (25th to 75th percentile). The line bisecting the box represents the median. The small square in the center is the mean, and whiskers indicate the 5th and 95th percentiles. We compare nonlabeled cells not exposed to and exposed to Colony Stimulating Factor-1 (CSF, controls), versus surface-labeled cells exposed to CSF (NHS-Cy5, cells metabolically labeled with N-azidoglucose (GlcNaz) or -mannose (ManNaz) conjugated to phos-DY650). As a control for the metabolically labeled cells, we have also included a DMSO-treated control. * $P < 0.0001$.

Figure 6. Modified macrophage homing and interaction with cancer cells. Time-lapse fluorescence microscopy images show migration and association of FITC-avidin labeled (in suspension) RAW 264.7 macrophages with MDA-MB-231 cancer cells at a 1:2 ratio across 0, 4, 8, and 12 h. Green = avidin-FITC macrophages; blue = MDA-MB-231 cells labeled with cell tracker dye. Scale bars indicate 100 μm . Video is available for viewing in Supporting Information Video 1.

Figure 7.

Macrophage biodistribution in an immune-competent mouse model of breast cancer. 4T1 cells (mouse mimic of stage IV human breast cancer) were orthotopically implanted into BALB/c mice. Macrophages were labeled via suspended methods with biotin/ avidin-Dy680 immediately prior to intravenous injection via tail vein. Following euthanasia at 4 and 24 h after injection, fluorescent imaging of organs was performed *ex vivo* using IVIS-CT.