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Tumor-Associated Macrophages Promote Invasion while Retaining Fc-Dependent Anti-Tumor Function

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Tumor-associated macrophages (TAMs) have been shown to promote tumor progression, and increased TAM infiltration often correlates with poor prognosis. However, questions remain regarding the phenotype of macrophages within the tumor and their role in mAb-dependent cytotoxicity. This study demonstrates that whereas TAMs have protumor properties, they maintain Fc-dependent anti-tumor function. CD11b⁺CD14⁺ TAMs isolated from primary human breast tumors expressed activating FcγRs. To model breast cancer TAMs in vitro, conditioned medium from breast cancer cells was used to drive human peripheral monocyte differentiation into macrophages. Tumor-conditioned macrophages were compared with in vitro derived M1 and M2a macrophages and were found to promote tumor cell invasion and express M2a markers, confirming their protumor potential. However, unlike M2a macrophages, tumor-conditioned macrophages expressed FcγRs and phagocytosed tumor cells in the presence of a tumor Ag-targeting mAb, unmasking an underappreciated tumoricidal capacity of TAMs. In vivo macrophage depletion reduced the efficacy of anti-CD142 against MDA-MB-231 xenograft growth and metastasis in SCID/beige mice, implicating a critical role for macrophages in Fc-dependent cell killing. M-CSF was identified in tumor-conditioned media and shown to be capable of differentiating macrophages with both pro- and anti-tumor properties. These results highlight the plasticity of TAMs, which are capable of promoting tumor progression and invasion while still retaining tumoricidal function in the presence of tumor-targeting mAbs. The Journal of Immunology, 2012, 189: 000–000.

Monocytes are capable of differentiating into macrophages with widely different properties depending on the external cues. Macrophages are also highly plastic and can rapidly alter their phenotype as local signals change (1). The classic proinflammatory macrophage subset, often termed M1 macrophages, is polarized by TLR2/4 ligands and the proinflammatory cytokine IFN-γ and mediates innate immunity. M1 macrophages secrete proinflammatory cytokines, promote Th1 responses through Ag presentation, have high expression of activating FcγRs, and are capable of tumor and tissue destruction (2, 3). At the other side of the spectrum are alternatively activated macrophages, broadly referred to as M2 macrophages, which can be differentiated in the presence of IL-4 and IL-13 (4, 5). In addition to IL-4/IL-13 macrophages (termed the M2a subset), more recent studies have implicated immune complexes (M2b) and IL-10 (M2c) as signals driving M2 macrophage polarization (6). M2 macrophages are associated with secretion of anti-inflammatory cytokines such as IL-10, and the production of chemokines that promote Th2 responses such as CCL-17 and CCL-22 (2). M2 macrophages have also been connected with immunoregulation and tissue remodeling. Over the years, significant effort has been focused on understanding how cues within the tumor microenvironment influence the properties of an additional subset of macrophages, termed tumor-associated macrophages (TAMs).

TAMs are often considered protumor due to a combination of properties resulting from their education within the tumor microenvironment (6, 7), and are broadly characterized as M2-like. TAMs support developing neoplasias by secretion of growth- and angiogenesis-promoting factors (7). TAMs also influence metastasis by aiding matrix breakdown and driving tumor-cell migration and invasion (7). In addition, TAMs are thought to suppress immune functions through a number of mechanisms. These include the secretion of anti-inflammatory cytokines such as IL-10, CCL22, and TGFβ (1, 7), which can downmodulate macrophage reactive oxygen intermediates (8, 9) and the recruitment of regulatory T cells to block cytotoxic T cell responses (10). Combined, these factors suggest that enrichment of TAMs within the tumor microenvironment supports tumor growth and metastasis. Indeed, in several human cancers, including breast, a high incidence of TAMs correlates with a poor clinical prognosis (7, 11).

Recent data have shown that clinical response rates to chemotherapies may be improved if immunogenic cell death mechanisms were also targeted (11, 12). One of the largest growth areas in the biotechnology and biopharmaceutical arena for cancer treatment is the development of anti-tumor mAb therapies (13). The mechanism of action(s) for anti-cancer Abs is typically very diverse and includes directly blocking target functionality, induction of apoptosis, and/or Fc-dependent recruitment of the immune system to promote tumor-cell destruction (14). Once a mAb binds to a tumor cell, the Fc portion of the mAb can recruit and activate components of the complement system and innate immune effector cells,
including macrophages, resulting in lysis and destruction of the mAb-targeted cancer cell through complement-dependent cytotoxicity (CDC), Ab-dependent cellular cytotoxicity, and/or Ab-dependent cellular phagocytosis (ADCP) (14, 15). In contrast to the role macrophages are thought to play in tumor promotion, numerous preclinical studies have implicated macrophages and engagement of FcγRs as critical for mAb-mediated tumor suppression in vivo (16–21). Similarly, a recent investigation has demonstrated that M2-like macrophages are capable of promoting robust ADCP in vitro (22). The traditionally accepted role of TAMs promoting tumor growth has also been challenged in the clinic where several clinical trials have shown that increased TAM infiltration correlated with a positive prognosis when the treatment regimen included a therapeutic mAb (23, 24).

The purpose of the current study was to reconcile these seemingly disparate properties of TAMs and to better understand the role of macrophages in mAb-dependent tumor cytotoxicity. Our results demonstrate that macrophages differentiated with conditioned media from a human breast cancer cell line (tumor-conditioned macrophages [TCM]) resembled breast TAMs isolated from fresh human tumors and displayed characteristics of both in vitro M1 and M2 macrophages. TCM promoted invasion of breast cancer tumor cell lines. In contrast, in the presence of an anti-tumor mAb, TCM differentiated similar to M1 macrophages and were capable of phagocytosing tumor cells in three-dimensional (3D) culture. To confirm the tumoricidal role of TAMs in vivo, we performed orthotopic xenograft studies in SCID/beige mice that retain macrophages as the primary functional immune effector cell (25, 26). The results demonstrated that an anti-tumor CD142 mAb was capable of suppressing tumor growth and metastasis in vivo, and that depletion of macrophages decreased the tumor-suppressive capacity of the mAb. These results demonstrate that TAMs have protumor properties in the absence of anti-tumor mAbs, but retain anti-tumor properties that can be used in the presence of an anti-tumor mAb.

Materials and Methods

Reagents

MDA-MB-231 cells were obtained from American Type Culture Collection and cultured in DMEM containing glutamax, 1× nonessential amino acids, and 10% FBS (Life Technologies). Human anti-CD142 IgG1 (CDR grafted, humanized) contains the same V region as the murine anti-human CD142 mAb TF8-5G9, which originated at the Scripps Research Institute and has been described previously (27–29). The generation and purification of single cleaved anti-CD142 with IgG-degrading enzyme of Streptococcus pyogenes were done, as described previously (29).

Clinical breast tumors

Human breast tumors (n = 9) were received within 24 h of surgical removal from each patient (BioOptions). Following resection, the tumors were shipped in RPMI 1640 containing 1-glutamine, 1× nonessential amino acids, and 10% FBS (Life Technologies). Human anti-CD142 IgG1 (CDR grafted, humanized) contains the same V region as the murine anti-human CD142 mAb TF8-5G9, which originated at the Scripps Research Institute and has been described previously (27–29). The generation and purification of single cleaved anti-CD142 with IgG-degrading enzyme of Streptococcus pyogenes were done, as described previously (29).

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In vitro macrophage differentiation

Human PBMCs were isolated from leukopaks (Biologics Specialty) using Ficol gradient centrifugation. CD14+ monocytes were purified from PBMCs by negative depletion using a CD14 isolation kit that did not deplete CD16+ monocytes (Stem Cell Technologies). Monocytes were plated at 0.1 × 106 cells/cm2 in X-VIVO-10 medium (Lonza) containing 10% FBS and under the following conditions: (M1) 25 ng/ml M-CSF (R&D Systems) for 7 d. A total of 50 ng/ml IFN-γ (R&D Systems) and 100 ng/ml LPS (Sigma-Aldrich) was added for the final 24 h of differentiation, (M2a) 20 ng/ml IL-4, and IL-13 (R&D Systems) for 7 d. (TCM) 50% day 4 conditioned media from MDA-MB-231 cells plated at 0.1 × 106 cells/cm2 in DMEM with 10% FBS, and (M-CSF) 25 ng/ml M-CSF for 7 d. For the experiments described in this work, different PBMC donors were used (FcγRIIA genotype R131/H131 or H131/H131 and FcγRIIIA genotype V158/V158 or F158/V158). Genotyping was performed by a commercial genotyping service (GNEWZ). Macrophages were photographed using a Nikon Eclipse TE2000-U microscope using a ×20 Hoffman modulation contrast objective (final original magnification of images ×200).

Flow cytometry

The following human Abs were obtained from BD Biosciences, unless otherwise noted: CD11b (ICRF44), CD16/FcγRIII (3G8), CD66b (1G05S), CD32a (IV.3; StemCell Technologies), HLA-DR (L243[G46-6]), CD86 (2331 [FUN-1]), CD64/FcγRI (10.1), DC-SEG (DCN46), CD206 (19.2), CD14 (Tuki; Life Technologies), and CD163 (RM3/1; BioLegend). The following mouse Abs were obtained from BD Biosciences, unless otherwise noted: CD45 (30-F11), CD11b (M1/70), Ly6G (IA8), F4/80 (BM8; BioLegend), CD16/32 (2.4G2), CD64 (X54-517.1), CD206 (MRSD3; BioLegend), I-A/E (M5/114.15.2), and Ly6C (HK1.4; BioLegend). Human anti-32b/FcγRIIb (2B6, mouse IgG2a) was constructed by gene synthesis, expressed, and purified (20, 31). In vitro differentiated macrophages (0.25 × 106 cells), clinical human breast tumors (1–3 × 106 cells), mouse PBMCs (0.5–1 × 106 cells), or MDA-MB-231 xenograft tumor cells (0.5–1 × 106 cells) were stained with fluorochrome-conjugated Abs in 7–10 multicolor flow cytometry panels for 30 min at 4°C. Prior to Ab staining, cells were stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (2:00, 15 min, incubation temperature; Life Technologies) in PBS and blocked with mouse anti-human CD45 or rat anti-mouse CD16/32 mAbs (1:50, 10 min, 4°C; both BD Biosciences). Washes between staining steps were done with BD Pharmingen Stain Buffer (BSA; BD Biosciences), and Ab-staining volumes were 100 μl in a 96-well U-bottom plate. Mouse isotype control Abs (IgG1, IgG2a, IgG2b, and IgM) were used in the following colors: Alexa Fluor 488, PE, PerCP Cy5.5, PE Cy7, Alexa Fluor 547, Alexa Fluor 647, Pacific Blue, BD Horizon V450 (all from BD Biosciences), and quantum dot 605-labeled mouse IgG2a (Life Technologies). Rat isotype control Abs (IgG1, IgG2a, IgG2b) were obtained from BD Biosciences, unless noted, in the following colors: Alexa Fluor 488, FITC, PE, Cy5.7, Alexa Fluor 647, APC, Pacific Blue, and Allophycocyanin, and Pacific Blue (BioLegend). Data acquisition for both human and mouse studies was performed on a LSR Fortessa (BD Biosciences), and analysis was done using FlowJo version 9.3.1 (Tree Star).

Luminex

Cytokine analyses were done using custom luminex kits purchased from Millipore (Milliplex map human cytokine/chemokine panels I–III). Sterile-filtered conditioned media was diluted 1:5 with X-VIVO-10 prior to analysis following kit protocol and measured on Bio Plex 200 instrument (Bio-Rad). All samples were run in triplicate.

Quantitative real-time PCR

RNA was isolated from previously snap-frozen cell pellets (0.5–2 × 106 cells) using RNaseasy Plus kit (Qagen). A total of 1.5 μg RNA was converted to cDNA in 60 μl reaction size using the High Capacity cDNA Reverse Transcription Kit containing RNaseP (Life Technologies). A quantity amounting to 4 μl cDNA product was amplified in 20 μl size quantitative PCRs using TaqMan Universal PCR Master Mix (1×) and TaqMan gene expression assay sets (1×) specific to human ALOX15 (Hs00937656_g1_), NOS2 (Hs01075529_m1_), and HPRT (4337368F) (Life Technologies). PCRs were run on an ABI 7900HT under standard cycling conditions. Normalized gene expression was calculated using 2−ΔΔCt method with hypoxanthine phosphoribosyltransferase as the endogenous control and a midrange M1 (ALOX15) or TCM (NOS2) samples as the calibrator (32). All samples were run in triplicate.

Three-dimensional culture

A total of 8 mg/ml Cultrex (Trevigen) was seeded (100 μl/well) to the bottoms of a 96-well black tissue culture-treated imaging plate (BD Biosciences) and allowed to solidify in the incubator for 2 h. MDA-MB-231- GFP, or breast epithelial cells (0.1 × 10^5) were seeded to the well center in 25 μl DMEM. Plates were incubated for 2 h in the incubator before being gently overlaid with 125 μl DMEM containing 2% Cultrex. Tumor spheroids were incubated for 4 d before 1.5 × 10^8 M1 macrophages, M2a macrophages, or TCM
were added in DMEM with 2% Caltrex. Cells were cocultured for 24 h prior to being photographed. Invasion was photographed using Nikon Eclipse TE2000-U epifluorescence microscopy. Multiple wells (>4) were set up per condition in each experiment, and at least three independent experiments were done using three different PBMC donors.

*Boydjen invasion assay*

MDA-MB-231 and indicated macrophages (2:1 ratio, 1.25 × 10⁵ total cells) were added to the upper chamber of a Matrigel-coated Transwell invasion assay plate (BD Biosciences) in X-VIVO-10 in serum-free media. Cells invaded through the Matrigel to X-VIVO-10 plus 10% FBS-filled bottom wells. After 22 h, the bottoms of the Transwells were stained with 4 μg/ml calcine AM in HBSS (without Mg²⁺ and Ca²⁺) in the incubator for 1 h. Fluorescence was read from the bottom of the plate with Spectrmax M5 plate reader at 494/517 nm (Ex/Em). All samples were run in triplicate in three independent experiments using macrophages generated from five independent PBMC donors.

*ADCP*

ADCP assays were performed, as previously described, using MDA-MB-231 cells stably expressing Turbo GFP (Sigma-Aldrich) (28) with some modifications. M1 and TCM were detected with CD11b (BD Biosciences; ICRF44) and CD14 (BD Biosciences; M5E2) coupled to Alexa Fluor 647 (Invitrogen), whereas M2a macrophages were detected with CD11b and CD206 (BD Biosciences; 19.2) coupled to Alexa Fluor 647. Percentage of phagocytosis was determined by the following equation: (100 × [%GFP⁻/AF647⁻]/[%GFP⁺ alone] + [%GFP⁺/AF647⁺]). Singlet gating was done, as shown in Supplemental Fig. 1C, to exclude doublet cell populations. As an additional control to ensure phagocytosis, following the 4-h ADCP incubation and macrophage marker labeling protocol (primary: unconjugated CD11b/CD14, and secondary: goat anti-mouse IgG [H + L chain] conjugated to Alexa Fluor 568 [Invitrogen]), the cells were seeded on a poly(t-lysine)-coated 96-well black imaging plate (10-20,000 cells/well) and visualized and photographed using a Nikon Eclipse TE2000-U epifluorescence microscope (×100) (Supplemental Fig. 1C).

*Three-dimensional ADCP*

A total of 8 mg/ml Caltrex was seeded (740 μl) to the bottom of a 24-well tissue culture-treated plate and allowed to solidify in the incubator for 2 h. A total of 6 × 10⁶ MDA-MB-231-GFP cells was seeded to the well center in 50 μl DMEM. Plates were incubated for 2 h in the incubator before being gently overlaid with 950 μl DMEM containing 2% Caltrex. Tumor spheroids were incubated for 4 d before 1 × 10⁴ TCM ± 2 μg/ml anti-CD11b and 25 μg/ml unconjugated CD14 and secondary: goat anti-mouse IgG [H + L chain] conjugated to Alexa Fluor 568 [Invitrogen]). The cells were seeded on a poly(t-lysine)-coated 96-well black imaging plate (10-20,000 cells/well) and visualized and photographed using a Nikon Eclipse TE2000-U epifluorescence microscope (×100). Macrophage depletion was maintained through the study with twice weekly 100 μl i.p. injections. On day 1, 2.5 × 10⁶ MDA-MB-231 tumor cells (50 μl, serum-free DMEM) were implanted into the #2 or #3 mammary fat pad. Intravenous Ab therapy was started on day 3, with half the groups receiving PBS at 5 mg/kg (100 μl/20 g) and the other half receiving anti-CD142 at 5 ml/kg (0.1 g/kg). Ab injections continued once weekly for the duration of the study. Tumor volumes were measured twice weekly and calculated as (L × W²)/2, in which “L” is the longest diameter and “W” is the shortest diameter of the tumor. ANOVA was used to compare the average day 42 values for the six treatment groups (SAS PROC GLM). The specific pairwise group comparisons of interest were estimated within the overall ANOVA model using contrasts; no α value adjustment for multiple comparisons was made. The in vivo study comparing the efficacy of anti-CD142, single-cleaved anti-CD142, and PBS was set up identical to above without cellular macrophage depletion (8 mice/group).

All animals were maintained in a facility approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture. The protocol was reviewed and approved by the Centrocon Institutional Animal Care and Use Committee.

At the end of the CEL study (day 42), the extent of macrophage depletion was evaluated in the tumor tissue, peripheral blood, and peritoneal cavity. Single-cell suspensions of the mouse tumors were generated using a human tumor dissociation kit, per manufacturer instructions (Miltenyi Biotech), before flow cytometry analysis. Mouse tumor DNA was isolated from each tumor using the DNeasy Blood and Tissue Kit (Qiagen). Extracted human DNA (metastatic cells) was amplified with primers and probe specific to human Alu elements (forward, 5′-CAT GGT GAA ACC CGG TCT CTA-3′; reverse, 5′-GCG TCA GCC TCC CCA GTA G-3′; probe, 5′-FAM-ATT AGC CGG TCG TGG CG TAMARA-3′). The probe was obtained from Life Technologies, and the primers from IDT. The amount of human DNA in each sample was normalized to overall mouse DNA in the lung sample by amplification with multiple of mouse β-actin (Life Technologies, MM00607939_s1). Each reaction (10 μl) contained ~200 ng lung DNA, TaqMan Universal PC master mix (1×; Life Technologies), and either mouse β-actin primer/probe set (1×) or 0.1 μM human alu forward primer, 0.1 μM human alu reverse primer, and 0.25 μM human alu probe. PCRs were run on an ABI 7900HT under standard cycling conditions. All data from the human Alu/mouse β-actin TaqMan assay were analyzed using the ΔCt method and displayed as 2⁻ΔΔCt (32).

*Results*

**Freshly isolated TAMs from human breast tumors express FcγRs**

To understand the potential role of macrophages in mAb-mediated tumor cell clearance, macrophages within human clinical breast tumors were characterized. Single-cell suspensions of freshly isolated human breast tumors (n = 9) were prepared to assess the FcγR expression profile of CD11b/CD14+ TAMs (35). Cell surface expression of FcγRs was detected in TAMs from a majority of the tumors analyzed. FcγRIIa was expressed on 98.1 ± 0.6% of TAMs found in the tumors analyzed, and FcγRI was expressed on 55.5 ± 10.4% of TAMs. FcγRIIB and FcγRIII were each expressed on a lower percentage of TAMs, 26.4 ± 8.7% and 14.2 ± 5.6%, respectively (Fig. 1A, 1B). The clinical characteristics of the individual tumor samples are summarized in Supplemental Table I. The flow cytometry gating strategy used to identify CD11b⁺CD14⁺ cells is shown in Supplemental Fig. 1A. CD11b⁺ cells made up 14.2 ± 2.3% of all live cells in the tumor, and CD11b⁺CD14⁺ cells were average of 0.7 ± 3.1% of all live cells (Fig. 1C, Supplemental Fig. 1B).
phages were differentiated by incubating monocytes with 25 ng/ml M-CSF, followed by polarization with 50 ng/ml IFN-γ and 100 ng/ml LPS for the final 24 h of culture (36). M2a macrophages were polarized with 20 ng/ml IL-4 and 20 ng/ml IL-13 (5). The morphology of the different in vitro differentiated macrophage subsets is shown in Fig. 1D. Both M1 macrophages and TCM had detectable levels of all three of the activating FcγRs (FcγRI, FcγRIIa, and FcγRIIb). The M2a macrophages did not express detectable levels of FcγRI, and had reduced levels of FcγRIIa and FcγRII compared with M1 and TCM (Fig. 1E). All three populations of macrophages had low (TCM) to barely detectable levels of the inhibitory FcγRIIb receptor (M1 and M2a macrophages). These results demonstrated that, similar to TAMs isolated from human breast tumors, both TCM and M1 macrophages expressed the activating receptors FcγRI and FcγRIIa.

**FIGURE 1.** TAMs from human breast tumors express FcγRs. (A) CD11b+CD14+ TAMs isolated from freshly obtained clinical tumor samples were analyzed by flow cytometry for surface expression of FcγRI/CD64, FcγRIIa/CD32a, FcγRIIb/CD32b, and FcγRIII/CD16. Two representative patients are shown of a total of nine tumors analyzed. Shaded histograms represent isotype control; (B) percentage of CD11b+CD14+ TAMs expressing FcγRI/CD64, FcγRIIa/CD32a, FcγRIIb/CD32b, and FcγRIII/CD16 compiled for all breast tumors analyzed (n = 9). Lines represent average expression percentage across all samples using gating strategy presented in (A); (C) Percentage of CD11b+ and CD11b+CD14+ cells in all live cells within human tumor samples. Different shapes represent individual patients and are consistent across all analyses (B, C); and (D, E) in vitro differentiated macrophages were photographed (original magnification ×200) on day 7 prior to being harvested and analyzed for surface expression of FcγRI/CD64, FcγRIIa/CD32a, FcγRIIb/CD32b, and FcγRIII/CD16 by flow cytometry. Representative flow cytometry plots are shown of more than three independent experiments with three different normal PBMC donors. Shaded histograms represent isotype control.

**TCM differentiated with breast tumor cell-conditioned media express FcγRs**

To model TAMs in vitro, human peripheral blood monocytes were differentiated into macrophages using conditioned medium from MDA-MB-231 breast cancer cells. For comparison, M1 macro-

**TCM express both M1- and M2a-associated markers**

We conducted further immunophenotyping to characterize the in vitro generated macrophages. All in vitro macrophages expressed CD11b, HLA-DR, and CD86. TCM expressed cell surface markers found on M2a macrophages, including DC-SIGN and the alternative activation marker, macrophage mannose receptor (CD206) (37), while also expressing markers similar to M1 macrophages, including CD14 (Fig. 2A). This marker profile pattern was also found on primary breast tumor CD11b+CD14+ TAMs, which also expressed HLA-DR, CD86, CD163, DC-SIGN, and CD206 to some extent (Fig. 2B, 2C). IL-4 and IL-13 have been shown in the literature to downregulate CD14 and increase HLA-DR in M2 macrophages, consistent to what was observed with our in vitro conditions (38, 39). Further characterization was done through analysis of the cytokines secreted from the three in vitro macrophage subsets. TCM secreted IL-6, IL-8, and CCL22 similar to M1 macrophages, but did not secrete TNF-α or IL-12, and lacked expression of inducible NO synthase (NOS2) (Fig. 2D). TCM also did not secrete CCL22 or express ALOX15, both of which were expressed by M2a macrophages (Fig. 2D, 2E). Thus, TCM differentiated with MDA-MB-231–conditioned media displayed characteristics of both M1 and M2a macrophages and resemble what was observed in CD11b+CD14+ TAMs isolated from patient breast tumors.

**TCM promote invasion of MDA-MB-231 cells**

One aspect of the protumor characteristics of TAMs is their ability to enhance tumor cell invasion (36). The invasion promotion abilities of TCM were compared with M1 and M2a macrophages. All three types of macrophages were incubated with MDA-MB-231 spheroids formed upon 3D culture in the basement membrane matrix, Cultrex, and the extent of tumor cell matrix invasion was assessed. As shown in Fig. 3A, M2a macrophages were potent inducers of tumor cell invasion after 24 h of 3D coculture. TCM promoted modest invasion over MDA-MB-231 cells alone, with M2a macrophages, promoting profile of the macrophage subsets was also demonstrated using Transwell invasion chambers in which coculture of individual macrophage subsets with MDA-MB-231 tumor cells increased cell invasion over MDA-MB-231 cells alone, with M2a macrophages, and TCM displayed more pronounced and significant invasion compared with M1 macrophages (Fig. 3B).

**TCM phagocytose breast tumor cells in the presence of an anti-CD142 mAb**

To characterize the role of TAMs in mAb-mediated cytotoxicity, the ability of different macrophage subsets to facilitate ADCP was...
assessed by coincubation of macrophages with MDA-MB-231 cells opsonized with an anti-CD142 human IgG1 mAb, and the percentage of phagocytosed MDA-MB-231 cells was determined by flow cytometry. This particular anti-CD142 mAb was chosen for these studies because it binds strongly to CD142 (tissue factor) expressed on the MDA-MB-231 cell surface, and its mechanism of action was shown to be mediated entirely through Fc-dependent killing. The importance of Fc-mediated tumor suppression in this model was confirmed by introducing a single proteolytic cleavage in the lower hinge of the anti-CD142 mAb. A single cleavage in the lower hinge has been shown previously to abrogate Fc-mediated cell-killing functions both in vitro and in vivo, without affecting the circulating \( t_{1/2} \) of the mAb (29). The intact anti-CD142 mAb was capable of suppressing MDA-MB-231 growth in SCID/beige mice, whereas the single-cleaved anti-CD142 mAb could not suppress tumor growth (Supplemental Fig. 2A). These results demonstrated the importance of Fc-dependent tumor suppression in this model system. For ADCP analysis, MDA-MB-231 cells were detected by their expression of GFP, and macrophages were detected through dual labeling of cell surface receptors expressed by each macrophage subset (i.e., Alexa Fluor 647-labeled anti-CD11b and anti-CD14 mAbs for M1 macrophages and TCM, and Alexa Fluor 647-labeled anti-CD11b and anti-CD206 mAbs for M2a macrophages). Control experiments determined that the markers used to detect the macrophages were not expressed on the MDA-MB-231 tumor cells, nor did MDA-MB-231 express any of the FcγRs (data not shown). Singlet gating and assessment of ADCP by fluorescence microscopy ruled out the
misclassification of tumor–macrophage interactions without internalization as true phagocytosis (Supplemental Fig. 1C, 1D). The population of cells representing MDA-MB-231 that had been phagocytosed by macrophages after a 4-h incubation was both GFP positive and Alexa Fluor 647 positive (Fig. 4A). As shown in Fig. 4B, both TCM and M1 macrophages were capable of robust ADCP, with maximum phagocytosis levels exceeding 50%. In contrast, the M2a macrophages displayed lower phagocytic potential. These data demonstrate that, similar to M1 macrophages, TCM are capable of robust ADCP, and that the ability of macrophages to phagocytose mAb-opsonized tumor cells correlated with the levels of activating FcγRs, in accordance with previous observations (22, 40).

**FIGURE 3.** TCM express FcγRs and promote tumor cell invasion. (A) MDA-MB-231 GFP spheroids were grown in 2% Cultrex for 3 d before the indicated day 7 macrophages were added. Representative fluorescent and bright field images (original magnification ×100) were taken to highlight tumor cell invasion induced by the presence of macrophages after 24 h of coculture of three independent experiments with three different donors. Green fluorescence represents GFP-positive MDA-MB-231 cells; and (B) invasion of MDA-MB-231 and indicated macrophages (2:1) in a Matrigel-coated Transwell invasion assay, as measured by number of calcin AM-detected cells on bottom of Transwell after 22 h. n = 5, run in triplicate, error bars represent ± SEM. **p < 0.01, ***p < 0.0001, unpaired two-tailed t test; GraphPad.

**FIGURE 4.** TCM and M1 macrophages can phagocytose Ab-bound tumor cells. (A) Representative flow cytometry plots depicting GFP-expressing MDA-MB-231 cells and human macrophages detected with CD11b and CD206 or CD14 conjugated to Alexa Fluor 647 in the presence of 9 or 0.00009 μg/ml anti-CD142 for 4 h. Data are representative of more than three separate experiments with three normal PBMC donors; (B) ADCP of MDA-MB-231-GFP cells by indicated macrophages in the presence of serial dilutions of anti-CD142 IgG1 for 4 h; and (C, D) MDA-MB-231 GFP spheroids were grown in 2% Cultrex for 3 d before day 7 TCM were added ± 2 μg/ml anti-CD142 mAb. Following 96 h of coculture, all cells were harvested from 3D culture, macrophages were detected with CD11b conjugated to Alexa Fluor 647, and ADCP was assessed by flow cytometry. Results presented are representative of three independent experiments using three different PBMC donors, and error bars represent ± SEM.

**TCM enhance tumor cell invasion and ADCP in 3D culture**

To more closely mimic the tumor microenvironment, we developed a culture system to assess macrophage ADCP of tumor cells grown in 3D spheroids. MDA-MB-231 cells formed 3D spheroids when grown in Cultrex for 4 d before the addition of TCM and/or an anti-CD142 mAb for 4 more days. In the absence of anti-CD142, TCM enhanced the invasiveness of the MDA-MB-231 3D spheroids (Supplemental Fig. 2B). To access the effect of addition of the mAb on macrophage Fc-dependent function, macrophages and tumor cells were harvested from 3D culture and analyzed for ADCP by flow cytometry. Coculture of tumor cells, TCM, and anti-CD142 resulted in phagocytosis of >60% tumor cells (Fig. 4C, 4D, Supplemental Fig. 2C). These results indicated that macrophages were capable of supporting tumor invasion (protumor) when coinubated with tumor cells alone, yet TCM were also capable of phagocytosing tumor cells (anti-tumor) when a tumor-targeting mAb was added to the coculture. Given the set-up conditions of the 3D tumor spheroid and macrophage coculture, the ratio of macrophages to tumor cells varied significantly from the conditions of the 4-h two-dimensional ADCP reaction, yet robust mAB-mediated phagocytosis was observed by TCM in both cases,
highlighting the potential anti-tumor capabilities of TAMs in a variety of tumorigenic settings.

**In vivo macrophages enhance tumor suppression by an anti-CD142 mAb**

To assess the contribution of TAMs to mAb-mediated tumor suppression in vivo, we used an orthotopic xenograft model with MDA-MB-231 tumor cells and the anti-CD142 mAb used above. To specifically assess the contribution of macrophages to tumor suppression, SCID/beige mice were chosen because they lack B cells, T cells, functional neutrophils, and functional NK cells, but retain macrophages (25, 26, 41). The importance of Fc-mediated tumor suppression in this model was detailed above (Supplemental Fig. 2A). To determine whether the Fc-dependent tumor suppression with anti-CD142 was mediated specifically by macrophages in this model, we performed macrophage depletion with CEL. Treatment with CEL depleted monocyte and macrophages from the peripheral blood and peritoneal cavity, and to a lesser extent within the tumors (Fig. 5A, Supplemental Fig. 2D, 2E) (42). Peripheral blood granulocytes were not depleted by CEL treatment, and their percentage within CD45⁺CD11b⁺ cells increased accordingly (Supplemental Fig. 2F) (42). This macrophage depletion resulted in a statistically significant reduction in the ability of anti-CD142 to inhibit MDA-MB-231 primary tumor growth and subsequent lung metastases (Fig. 5B–D). These results confirm that macrophages are a critical mediator of in vivo mAb Fc-dependent tumor suppression, as well as mAb-mediated metastasis prevention.

**Tumor-secreted M-CSF generates macrophages with pro- and anti-tumor properties**

To determine what was driving the differentiation of macrophages that displayed both pro- and anti-tumor characteristics, the levels of various cytokines were measured in MDA-MB-231–conditioned media through luminex analyses. High levels of M-CSF (69 ± 23 ng/ml) were detected in the MDA-MB-231–conditioned medium, as well as lower levels of GM-CSF, IL-6, IL-8, CCL2, and vascular endothelial growth factor (VEGF) (Fig. 6A). Levels of IL-10, IL-4, IL-13, and IFN-γ were undetectable (data not shown). The cytokine/chemokine profiles secreted from freshly resected human breast tumors after incubation in medium for 24 h were also assessed. The results revealed detection of high levels of M-CSF (6.6 ± 3.3 ng/ml) and lower levels of CCL2, VEGF, IL-8, and IL-6 secretion from patient tumors (Fig. 6B), comparable to what was detected in the MDA-MB-231–conditioned medium. To assess the impact of M-CSF on macrophage differentiation, macrophages were generated from human peripheral blood monocytes cultured for 7 d with 25 ng/ml M-CSF and compared with the other subsets of macrophages. As shown in Fig. 6C, M-CSF macrophages promoted tumor cell 3D invasion similar to M2a macrophages. However, M-CSF macrophages also expressed FcγRs and could phagocytose tumor cells similar to TCM (Fig. 6D–F). Further comparison of M-CSF macrophages with TCM revealed that M-CSF macrophages did not express DC-SIGN or CD206, nor did they secrete IL-6, IL-8, CCL22, or TNF-α in a similar pattern as TCM (Fig. 6G, 6H, Supplemental Fig. 3A–C). Thus, M-CSF was sufficient to generate macrophages that could promote tumor cell invasion and ADCP, but additional factors (such as VEGF, IL-8, CCL2, IL-6) secreted from tumor cells may have also contributed to the overall TAM phenotype observed in MDA-MB-231–conditioned media–derived macrophages.

**Discussion**

Defining the role of macrophages within the tumor microenvironment has proven challenging due to the high level of macrophage functional plasticity, which is influenced by the multitude of different cytokines and chemokines present within the tumor microenvironment (7, 43). The tumor-promoting potential of macrophages has been demonstrated through effects on tumor cell invasion, promotion of angiogenesis, and inhibition of immune responses (7). In contrast, multiple preclinical tumor models have demonstrated a key role for macrophages in mAb-mediated tumor
mAbs mediate their therapeutic effect through target inhibition and/or Fc-mediated immune effector functions. The nature of FcγR expression in patients treated with anti-tumor mAbs has received much attention recently because several clinical trials have documented that patients with high-affinity polymorphisms of FcγRIIa and FcγRIIIa had increased progression-free survival (44–46), and these observations are often used to argue for the importance of Fc-mediated immune effector function in anti-cancer mAb therapeutics (15). Many in vitro studies have implicated Ab-dependent cellular cytotoxicity mediated by NK cells as the mechanism of action of immune effector-mediated tumor cell killing by current clinically approved mAb therapies used in oncology (14, 47, 48). Unlike NK cells, which only express FcγRIIa, monocytes and macrophages can express all three activating receptors as well as the inhibitory FcγRIIb (47). Several cytokines can increase FcγR expression on monocytes/macrophages, including IFN-γ alone or in combination with TLR ligands, as well as M-CSF alone or in combination with IL-10 (22, 49, 50). Increased FcγR expression on macrophages typically correlates with mAb-mediated cell-killing functions (15, 22, 40). In contrast, IL-4 can decrease FcγR expression, leading to decreased MAb-mediated cytotoxic capabilities (38). In our studies, M2a macrophages differentiated in the presence of IL-4 and IL-13 had low FcγR expression (Fig. 1E) and low ADCP potential (Fig. 4A, 4B), whereas M1 macrophages had high FcγR expression (Fig. 1E) and potent ADCP capabilities (Fig. 4A, 4B). TCM differentiated with tumor cell line-conditioned medium were similar to M1 macrophages in terms of FcγR expression and ADCP. Others have used tumor cell line-conditioned supernatants to drive monocyte to macrophage differentiation, and cell viability is often dependent on the presence of GM-CSF or M-CSF (36). The MDA-MB-231–conditioned supernatants were tested for the presence of several cytokines, and the levels of M-CSF were the highest (Fig. 6A). Culturing monocytes in the presence of M-CSF alone resulted in macrophages that expressed activating FcγRs, were capable of ADCP, yet still promoted tumor invasion in 3D cultures. These observations highlight the complexities associated with the influence of M-CSF on macrophages. Expression of M-CSF in the tumor microenvironment can increase the recruitment of monocytes/macrophages, which is often correlated with poor clinical outcome (51). However, as discussed above, M-CSF can augment FcγR expression and tumor-killing capacity in the presence of tumor-targeting mAbs (Fig. 6).

FIGURE 6. M-CSF in tumor cell-conditioned media generates macrophages that are pro- and anti-tumor. (A) Luminex analysis for indicated cytokines in day 4 conditioned media from MDA-MB-231 cells (n = 3, run in triplicate, error bars represent ±SEM); (B) Luminex analysis for indicated cytokines secreted from clinical human breast tumor samples (n = 9, run in triplicate, error bars represent ±SEM). Different shapes represent individual patients and are consistent across each cytokine measured and as shown in Figs. 1B, 1C, and 2C; (C) MDA-MB-231 GFP spheroids were grown in 2% Cultrex for 3 d before M-CSF–treated and M2a (IL-4/IL-13) day 7 macrophages were added. Two representative bright field images (original magnification ×100) were taken to highlight tumor cell invasion induced by the presence of each macrophage subset after 24 h of coculture; (D) M-CSF macrophages were analyzed for surface expression of FcγRI/CD64, FcγRIIa/CD32a, FcγRIIb/CD32b, and FcγRIII/CD16 by flow cytometry; (E) representative flow cytometry plots depicting GFP-expressing MDA-MB-231 cells and day 7 M-CSF macrophages detected with CD11b and CD14 conjugated to Alexa Fluor 647 in the presence of 9 or 0.00009 μg/ml anti-CD142; (F) ADCP of MDA-MB-231-GFP cells by indicated macrophages in the presence of serial dilutions of anti-CD142 IgG1; (G) cell surface expression of DC-SIGN and CD206 in M-CSF macrophages as measured by flow cytometry (n = 3); and (H) Luminex analysis of indicated cytokines secreted into day 7 in vitro M-CSF–differentiated macrophage-conditioned media (n = 3, run in triplicate, error bars represent ±SEM).
rectly to 3D cocultures can promote TCM phagocytosis of tumor cells in the 3D matrix. The use of this assay system uniquely provided an opportunity to determine that the same population of TCM has both protumor (supporting invasion) and anti-tumor (promoting ADCP of mAb-opsonized tumor cells) properties.

The tumor-promoting role of macrophages in vivo has also been well documented. Previous studies have shown that in vivo depletion of macrophages using CELs reduced tumor growth, presumably due to the loss of proangiogenic factors secreted by the macrophages (7). In contrast to studies addressing the protumor capabilities of TAMs, numerous investigations into the mechanism of action of anti-tumor mAbs have specifically implicated macrophages as necessary for the immune-mediated tumor-suppressing effects (16–21). Olfazoglu et al. (18) have previously shown that neither neutrophil nor NK cell depletion altered the therapeutic benefit of the lymphoma tumor-targeting anti-CD40 Ab SGN-40, whereas macrophage depletion blocked mAb-mediated tumor inhibition of non-Hodgkin’s lymphoma cells. The importance of monocytes and macrophages in tumor-targeting anti-CD20–mediated tumor suppression has also been documented (16, 20, 48, 55). We sought to test the role of macrophages in vivo using a xenograft model system that required Fc functionality for efficacy. Growth inhibition mediated by an anti-CD142 therapeutic mAb of breast tumor xenografts was decreased upon single cleavage in the lower hinge of the mAb and upon macrophage depletion, highlighting a clear role for these cells in the Fc-mediated mAb mechanism of action. In addition, a profound effect on mAb-mediated inhibition of lung metastasis was observed upon macrophage depletion in our study (Fig. 5D), but no effect on lung colonization by metastatic cells was observed upon macrophage depletion. Previous work by multiple investigators has shown that macrophages often help facilitate the metastasis of breast cancer cells to the lung, particularly in the PyMT spontaneous breast cancer model in which metastases were reduced upon genetic and therapeutic depletion of macrophages (51, 56). Others have reported a role for granulocytes in metastatic seeding and homing to particular organs (42). In our in vivo study, there was no significant difference in the amount of metastatic cells plus/minus macrophage depletion, and the granulocyte population was not depleted by the CEL treatment, providing evidence that, in this model system, granulocytes may play more of a role in metastatic seeding than macrophages in the absence of tumor-targeting mAbs (Fig. 5D and Supplemental Fig. 2E). Importantly, with this report, we demonstrate that, in the presence of a therapeutic mAb, macrophages can be essential for inhibition of metastasis.

In our studies, clodronate-mediated depletion of macrophages did not result in a complete loss of function of the anti-CD142 mAb. Although macrophages are considered the main effector cell in SCID/beige mice, other Fc-dependent effects may be present. CDC is one explanation for the partial effect, although the potency of CDC in the solid tumor microenvironment by mAb-based monotherapies has been questioned (57). It was previously demonstrated that mouse serum was not capable of eliciting CDC with a tumor-targeting mAb with a human IgG1 Fc (58). Therefore, CDC may not have been a main contributing factor for the partial efficacy in the macrophage depletion study. Another potential explanation is incomplete CEL depletion of macrophages, particularly for intratumoral TAMs. Vonderheide and colleagues recently used CEL depletion to show the importance of CD40-activated macrophages in an anti-CD40 agonist mAb-mediated tumor suppression model (59). CEL treatment reduced the efficacy of mAb treatment; however, CEL treatment did not deplete intratumor macrophages. In our system, tumor-resident TAMs resistant to CEL depletion may have also contributed to the incomplete loss of function (Supplemental Fig. 2F).

The studies presented in this work demonstrate the importance of macrophages in suppressing both xenograft tumor growth as well as suppression of tumor cell metastasis to the lung. The existence of a well-established orthotopic xenograft model enabled these analyses. However, it has become increasingly apparent that extensive signaling networks exist between immune cells, tumor cells, and the tumor stroma (reviewed in (7) and others). These complex interactions are best modeled in fully immune competent mice where tumors arise spontaneously. At this time, these systems have not been adapted to widely study the mechanisms of action for tumor-targeting mAbs. Establishment of these model systems with well-defined tumor-targeting mAbs will help to further disseminate the role that macrophages play within the tumor microenvironment and how tumor-targeting mAbs can best be designed to exploit the anti-tumor potential of TAMs.

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Disclosures

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