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High-density gene expression analysis of tumor-associated macrophages from mouse mammary tumors

Tumorigenesis and Neoplastic Progression

High-Density Gene Expression Analysis of Tumor-Associated Macrophages from Mouse Mammary Tumors

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Clinical and experimental evidence indicates that tumor-associated macrophages (TAMs) promote malignant progression. In breast cancer, TAMs enhance tumor angiogenesis, tumor cell invasion, matrix remodeling, and immune suppression against the tumor. In this study, we examined late-stage mammary tumors from a transgenic mouse model of breast cancer. We used flow cytometry under conditions that minimized gene expression changes to isolate a rigorously defined TAM population previously shown to be associated with invasive carcinoma cells. The gene expression signature of this population was compared with a similar population derived from spleens of non-tumor-bearing mice using high-density oligonucleotide arrays. Using stringent selection criteria, transcript abundance of 460 genes was shown to be differentially regulated between the two populations. Bioinformatic analyses of known functions of these genes indicated that formerly ascribed TAM functions, including suppression of immune activation and matrix remodeling, as well as multiple mediators of tumor angiogenesis, were elevated in TAMs. Further bioinformatic analyses confirmed that a pure and valid TAM gene expression signature in mouse tumors could be used to assess expression of TAMs in human breast cancer. The data derived from these more physiologically relevant autochthonous tumors compared with previous studies in tumor xenografts suggest tactics by which TAMs may regulate tumor angiogenesis and thus provide a basis for exploring other transcriptional mediators of TAM trophic functions within the tumor microenvironment. *(Am J Pathol 2009, 174:1048–1064; DOI: 10.2353/ajpath.2009.080676)*

In many human cancers, a high density of tumor associated macrophages (TAMs) correlates with poor prognosis.[1] This is particularly true in breast cancer where the greatest numbers of studies have been performed.[2] The overexpression of macrophage growth factors and chemoattractants similarly correlates with poor prognosis. In human studies, overexpression of the primary macrophage growth, proliferation and differentiation factor, colony-stimulating factor-1 (CSF-1) correlates with poor prognosis in ovarian, breast and endometrial cancer, among others.[3–6] CCL2 (MCP-1) is another example of a macrophage chemokine that is over-expressed in breast tumors[7,8] and whose expression correlates with accumulation of TAM and significantly poorer prognosis.[9] Taken together, these human studies illustrate the active recruitment of macrophages to a growing tumor, and furthermore suggest that in breast cancer, the presence of a high density of these TAMs facilitate tumor progression to malignancy.

Experimental studies in mouse models of breast cancer performed by our laboratory and others have provided support for this conclusion. One model in which the polyoma middle T (PyMT) oncoprotein is expressed in the mammary epithelium directed by the mouse mammary tumor virus (MMTV) long terminal repeat is a reliable mouse model for human breast cancer. These animals demonstrate spontaneous hyperplastic lesions at around 8 weeks of age that progress to late-stage metastatic malignancy through numerous stages reminiscent of human mammary adenocarcinoma.[10] When these mice were crossed to mice lacking CSF-1 ($Csf1^{op/op}$) the resulting female offspring displayed severely diminished

macrophage recruitment to tumors, and progression to late-stage malignancy was significantly delayed with metastasis dramatically reduced.[11] Restoration of CSF-1 to the mammary fat pad restored tumor progression and metastatic capability. It has been further elaborated that restoration of the pro-angiogenic factor, vascular endothelial growth factor (VEGF)-A to the tumor microenvironment in these macrophage-depleted animals restores tumor progression[12] suggesting one mechanism by which TAMs facilitate tumor progression: through regulation of tumor angiogenesis.[13] Similarly, depletion of CSF-1 or CSF-1 receptor signaling in host macrophages reduced tumor growth in a xenograft model of human breast cancer and this was associated with reduced angiogenesis.[14]

Animal models have revealed further insight into functional mechanisms by which macrophages promote tumor progression. At least six traits have been identified that macrophages can confer on tumors that promote their progression to malignancy.[15] For example in addition to tumor angiogenesis as mentioned above, it is known that macrophages are potent sources of metalloproteases that aid in matrix degradation[16] and can facilitate efficient intravasation of carcinoma cells from a primary tumor into the surrounding stroma. Furthermore, macrophages can induce tumor cell migration through the stroma and enhance intravasation via the production of growth factors such as epidermal growth factor (EGF) in a reciprocal chemotactic loop with tumor-produced CSF-1[17,18] or via the production of Wnt ligands.[19] It is speculated that these tissue trophic functions of TAMs may recapitulate functions of macrophages during development, such as regulation of epithelial cell movement in terminal end buds during mammary gland development.[20,21] This contrasts with the classical view that macrophages are critical mediators of innate immunity by phagocytosing pathogens that are harmful to the host, and promoting adaptive immunity through antigen presentation, giving them the capability to reject tumors expressing foreign antigens. However, it is now understood that in the tumor microenvironment, TAMs exhibit decreased capacity to facilitate carcinoma cell killing and inhibit adaptive immune responses, a process that allows the tumor to evade T-cell mediated tumoricidal activity.[22,23]

In recognition of the number of functions attributed to the TAM that can result in tumor promotion plus the apparent ability of the tumor microenvironment to educate the macrophages to have functions that enhance tumor survival and growth,[24] the goal of this study has been to use high density gene expression arrays to profile TAMs from the late stage primary mammary tumors from the PyMT mouse model of breast cancer to define a transcriptome that could confer these functions. A rigorous definition of our isolated macrophage population, paired with methods to minimize perturbations in gene expression, ensured the transcriptome defined was representative of TAMs. This TAM population was then compared with a classical "immune" population with the same characteristics isolated from spleens of non-tumor bearing mice. Various bioinformatic approaches revealed a transcriptome that emphasized tissue trophic functions and particularly angiogenic mediators. We hypothesize that the data from these microarrays can be further explored for novel therapeutic targets against many other tumor-promoting activities of TAMs.

Materials and Methods

Mice

All procedures involving mice were conducted in accordance with National Institutes of Health regulations concerning the use and care of experimental animals. The study of mice was approved by the Albert Einstein College of Medicine animal use committee. The FVB/N-Tg(MMTV-*PyVmT*)*Mul* (PyMT) transgenic mice were kindly provided by Dr. W.J. Muller (McGill University, Canada) and have been described previously.[10,25] *Tg(Csf1r-Gfp)Hume* (*MacGreen*) mice have also been described previously.[26] Male PyMT mice on an FVB background were bred to homozygous MacGreen female mice on a mixed background to generate PyMT mice that produce tumors with green fluorescent protein-labeled macrophages. All genotyping was done by PCR. Tumors were allowed to grow until 14 to 16 weeks to ensure late-stage carcinomas for TAM isolation. Splenic macrophages were isolated from littermates that do not carry the PyMT transgene.

Fluorescent Activated Cell Sorting

Flow cytometry was used for two purposes in this study: to cell sort TAM and splenic macrophages and to immunophenotype TAM. To identify phagocytic cells, 12 to 14-week-old PyMT/MacGreen female mice were lateral tail-vein injected with 200 μl 10 mg/ml 70,000 MW dextran conjugated to the Texas Red fluorophore (Invitrogen, Eugene, OR) resuspended in PBS. Two hours postinjection, animals were anesthetized with isoflurane and then perfused i.c. with ice cold PBS. Following sacrifice, all subsequent steps were performed at 4°C.

Isolating Macrophages

To avoid loss of surface markers, tumors and spleen were minced and filtered four times through graded nylon filters, centrifuged at 1200 RPM for 5 minutes and then resuspended in erythrocyte lysis buffer (Beckman-Coulter, Marseille, France). Cells were washed three times in nuclease-free PBS containing 2% bovine serum albumin (PBS + 2% BSA). Texas Red/EGFP double-positive cells were sorted on a DakoCytomation MoFlo High-Speed Cell Sorter (DakoCytomation, Inc, Fort Collins, CO) at 23 p.s.i. into PBS + 2% BSA.

Immunophenotyping TAMs

Tumor was minced in 1 ml of αMEM medium before adding Liberase at 0.028 Wunsch units/ml (Roche, Indianapolis, IN) and DNase I at 20 μg/ml (Sigma-Aldrich, St. Louis, MO) as previously described.[12] The mixture was incubated for 30 minutes at 37°C under gentle agitation.

Digestion was stopped with 0.5 ml fetal bovine serum (FBS) and 100 μl 0.5M EDTA pH 8.0. The suspension was serially filtered as described above, erythrocyte lysis was performed and cells were re-suspended in PBS + 2% bovine serum albumin (BSA) and incubated with Fc receptor block using rat mAb anti-mouse CD16/CD32 (BD Biosciences, San Jose, California) for 10 minutes. Subsequently cells were incubated with phycoerythrin (PE)-Cy5 conjugated anti-mouse F4/80, allophycocyanin-conjugated anti-mouse Gr1, PE-Cy7 conjugated anti-mouse CD11b, or PE-conjugated anti-mouse CD115/CSF1R (all eBioscience, San Diego, California) for 40 minutes in the dark. Samples were washed, fixed, and analyzed on DakoCytomation Mo-Flo to detect enhanced green fluorescent protein (EGFP), Texas Red, PE-Cy5, allophycocyanin, PE-Cy7, and PE.

Cytospin

Texas-Red$^+$/GFP$^+$ and Texas-Red$^-$/GFP$^+$ cells from tumor cell suspension (as described for fluorescent-activated cell sorting) were sorted separately into PBS + 2% BSA. Sorted cells were pelleted and resuspended into 100 μl PBS + 2%BSA and cytospun onto Colorfrost/*Plus* Microscope Slides (Fisher), followed by fixation in methanol for 5 minutes. Slides were briefly air-dried then stained with Accustain Wright-Giemsa Stain (Sigma-Aldrich, St. Louis, MO) for 5 minutes. Excess stain was rinsed with deionized water, dried, and mounted.

Immunohistochemistry

Primary tumors from late-stage tumor bearing animals were dissected and frozen into optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan). Tissues were serially sectioned at 7 μm by cryostat and then prepared for immunohistochemistry. In brief, following dehydration, sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase activity. Sections were blocked in normal rabbit serum for 10 minutes, followed by incubation with primary antibody for 1 hour at room temperature in a humidified chamber. The following primary antibodies were used: rat mAb to mouse F4/80 (Caltag Laboratories Inc., Burlingame, CA), rat mAb to mouse Gr1 (BD Pharmingen, San Jose, CA), and rat mAb to mouse clone 7/4 (Caltag Laboratories Inc.) for macrophage, myeloid, and neutrophil detection, respectively. Sections were next incubated in rabbit-anti-rat secondary antibody for 40 minutes at room temperature in a humidified chamber. Specific reactivity was detected using a peroxidase-based detection kit (Vector Laboratories, Burlingame, CA) as previously described.[10]

Immunofluorescence

As previously described,[27] tissue from MacGreen primary tumors with or without Texas-red dextran i.v. injection were dissected and fixed in 5% formalin in 20% sucrose/PBS for 24 hours at 4°C followed by freezing and sectioning. In the dark, sections were washed with deionized water and blocked for 1 hour with 10% goat serum.

Sections were incubated in the dark at 4°C for 12 hours with primary antibodies F4/80, Gr1 (listed above) and anti-mouse CD115/CSF-1R (kindly provided by E.R. Stanley, AECOM). Next, tissue sections were incubated with Alexa Fluor 568 conjugated goat anti-rat antibody (Invitrogen, Carlsbad, CA) for 1 hour and then stained with 0.3 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) for five minutes followed by wash and mounting.

RNA Extraction, Amplification, and cDNA Preparation

Total RNA was extracted from fluorescent-activated cell-sorted TAMs and splenic macrophages using RNeasy Micro Kits (Qiagen, Valencia, CA) according to the manufacturer's instruction. Amplification-grade DNase 1 treatment was performed on the RNA elution column to remove potential genomic DNA contamination. Approximate yields were 150 ng; quality was determined using a nano-biosizing assay (Agilent Bioanalyzer; Agilent Technologies, Palo Alto, CA).

Two hundred ng of RNA from samples was resuspended into 11 μl of RNase/DNase-free water, and a single round of linear amplification was performed by the *in vitro* transcription T7 promoter method as outlined by the manufacturer's protocol (Ambion's Message Amp T7 Kit; Ambion, Austin, TX). For microarray samples, a second round of linear amplification was performed with 200 ng of first round amplified material. At all steps, yield and quality were established using spectrophotometry and an Agilent Bioanalyzer.

For samples to be used for microarray hybridization, Superscript III (Invitrogen) reverse transcriptase was used to prepare 5 μg cDNA from amplified RNA. Random primers (Invitrogen) were used to prime reactions. Second-strand cDNA synthesis was performed using *E. coli* DNA ligase (Invitrogen), *E. coli* DNA polymerase 1 (Invitrogen), and T4 DNA polymerase (Invitrogen). RNase H (Invitrogen) treatment was additionally performed. The reaction was stopped with 0.5 M/L EDTA and then purified using a PCR purification kit (Qiagen) following manufacture's protocol. Samples were resuspended to approximately 200 ng/μl.

Gene Expression Arrays

Five micrograms of double stranded cDNA from each TAM and splenic macrophage sample were used for gene expression array processing. The expression array chip used contained 385,000 60-mer probes representing 42,586 genes (average nine probes per target) (NimbleGen, Reykjavik, Iceland). A total of four independent samples for each macrophage population were prepared. At NimbleGen, quality and yield were verified before DNA end-labeling, hybridization, and scanning. Raw data files for each sample were normalized, background-corrected and saved to logarithmic scale using a Robust Multi-Array Analysis[28] as implemented by NimbleScan software, version 2.2.33. Normalized data were analyzed and presented using R project (*http://www.R-project.org*).[29,30] All samples fulfilled quality criteria as determined by generation of pair-wise scat-

Table 1. Quantitative rtPCR Primer Sequences

Symbol	Name	Genbank Accession	Forward primer	Reverse primer
ADAM8	A disintegrin and metallopeptidase domain 8	NM_007403	5′-AGCCTGCCAGCTAAGAACAG-3′	5′-AACTGGGAGTGGTGAACTGG-3′
APOE	Apolipoprotein E	BC083351	5′-GGTTCGAGCCAATAGTGGAA-3′	5′-GGTGATGATGGGGTTGGTAG-3′
ARG1	Arginase 1, liver	NM_017134	5′-CAGATATGCAGGGAGTCACC-3′	5′-CAGAAGAATGGAAGAGTCAG-3′
C3AR1	Complement component 3a receptor 1	BC003728	5′-ATTGGGACTGCTAGGCAATG-3′	5′-GGTGAGATGGAGGAACCAGA-3′
CXCL4	Chemokine (C-X-C motif) ligand 4	NM_019932	5′-AGTCCTGAGCTGCTGCTTCT-3′	5′-GATCTCCATCGCTTTCTTCG-3′
ECM1	Extracellular matrix protein 1	NM_007899	5′-CTCCGAGTTGACCACTCTGTAA-3′	5′-TCGTACACAGGGATGTCTTCTG-3′
IL18	Interleukin 18	AY157834	5′-GACTGGCTGTGACCCTCTCT-3′	5′-GATGAATTGGCGTGGAATCT-3′
IRF4	Interferon regulatory factor 4	NM_013674	5′-AGCTCATGTGGAACCTCTGC-3′	5′-TGGTTCATCCAGCTGACTTG-3′
ITGA6	Integrin, alpha 6	NM_008397	5′-CAGGTTGTGGAACAGCACAT-3′	5′-GCGTGAGGGAGCTTGATATT-3′
ITGB7	Integrin, beta 7	NM_013566	5′-CAACTGGAAGCAGGACAACA-3′	5′-AGTCTGCTTCCCTGGTCAGA-3′
LTBP3	Latent transforming growth factor beta binding protein 3	NM_008520	5′-TCCCCTTTCAGACCAGTGAG-3′	5′-CCTGGTCCTGTCTTCTCTCG-3′
MIP1A	Macrophage inflammatory protein 1 alpha (chemokine [C-C motif] ligand 3)	NM_011337	5′-ACCAATGACACTCTGCAACCA-3′	5′-GATGAATTGGCGTGGAATCT-3′
MMP12	Matrix metallopeptidase 12	NM_008605	5′-TGATGCAGCTGTCTTTGACC-3′	5′-CCTGGGAAGTGTGGAAAT-3′
STAB1	Stabilin 1	NM_138672	5′-GTTTGTCACTCACACACCCTGT-3′	5′-ATAGCGGCAGTCCAGAAGTATC-3′
VEGFA	Vascular endothelial growth factor alpha	NM_009505	5′-CAGGCTGCTGTAACGATGAA-3′	5′-GCATTCACATCTGCTGTGCT-3′

ter, MA [the log ratio of intensities of two dyes used in the hybridization, Cy3 and Cy5 (M), to the average of the log intensities (A)] and expression density plots. Students' two-tail *t*-tests were conducted between the TAM and splenic macrophage samples for each transcript and fold-change was determined. Transcripts whose abundance were significantly altered ($P < 0.05$) and an absolute fold change greater than 2 were defined as differentially regulated. Using these criteria, 831 transcripts were called as down-regulated; 926 as up-regulated. Significance Analysis of Microarray (SAM) analysis[31] was used for more stringent gene selection criteria. A delta value of 1.74 called 462 significantly regulated transcripts with a false discovery rate of 10%. Hierarchical clustering[32] was performed using MultiExperiment Viewer version 4.1.01 (*http://www.tm4.org*).

Quantitative Real-Time PCR

For samples to be used for qrtPCR, Superscript III (Invitrogen) reverse transcriptase was used to prepare 200 ng cDNA from amplified RNA. Random nonamers (kind gift from Dr. Sumanta Goswami, Yeshiva University) were used to prime the reaction. Relative transcript abundance was detected by SybrGreen (Applied Biosystems, Foster City, CA) on the ABI 7900HT thermal cycler using gene-specific primers (Table 1). Gene expression was normalized to the housekeeping gene, cyclophilin A (*Ppia*), and expressed values in TAM relative to control splenic macrophages were determined using the $\Delta\Delta$CT method.[33]

Bioinformatics

The Ingenuity Pathways Knowledge Base (IPA) was used to identify enriched cellular and molecular functions among differentially regulated transcripts. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (*http://david.abcc.ncifcrf.gov*), the Gene Ontology Project, (*http://www.geneontology.org*) and extensive literature review was used for annotating regulated transcripts with a gene ontology designation for pie-chart analysis. Fetal macrophage gene expression data[34] was downloaded and processed as per a recent subsequent study.[35] Microsoft Office Access 2007 was used to associate the data with TAM gene expression data. Fisher's exact *t*-test was used to assess significance of association between datasets. Oncomine (*http://www.oncomine.org*), was used to mine human breast cancer microarray data as previously described.[36,37]

Bone Marrow-Derived Murine Macrophage Cultures

Bone marrow-derived murine macrophages (BMMs) were prepared as previously described.[38] Met-1[fvb2] cells
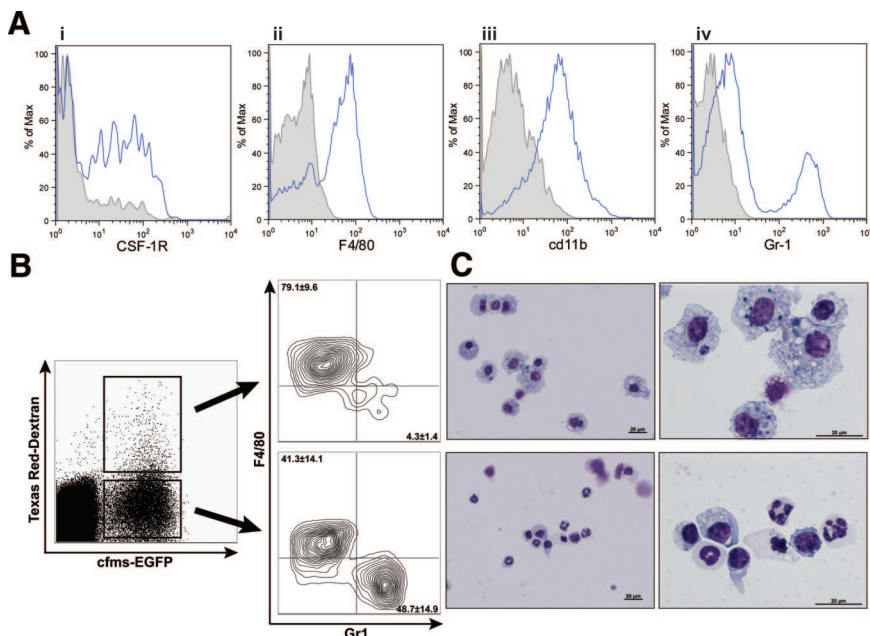
Figure 1. Csf1r-EGFP expression and Texas-Red conjugated 70kd dextran identify a pure population of TAMs *in vivo*. **A:** Single-cell suspensions were prepared from late-stage MacGreen PyMT tumor bearing females and stained for CD115 (external epitope of CSF-1R) (i); F4/80 (ii); CD11b (iii); Gr1 (iv). Shaded gray histograms indicate unstained EGFP[+] control. **B:** Single cell suspension of tumor from late-stage MacGreen PyMT tumor I.V. injected with texas-red conjugated dextran was prepared and analyzed by flow cytometry. Dextran[+]/EGFP[+] (**top panel**) and dextran[−]/EGFP^{+} cells (**bottom panel**) were separately analyzed for expression of F4/80 and Gr1. Average percentages with SEM of three experiments are indicated. Shown are representative plots. **C:** Cytospin followed by Wright-Giemsa staining was performed on dextran[+]/EGFP[+] and dextran[−]/EGFP^{+} cells sorted by flow cytometry. Dextran[+]/EGFP[+] (top panels) cytospin confirms a pure population of mononuclear phagocytes whereas dextran[−]/EGFP[+] (**bottom panels**) reveal a heterogeneous population of mononuclear and polymorphonuclear cells. Scale bar = 20 μm.

originally derived from mammary carcinomas in FVB/N-Tg(MMTV-*PyVmT*)[39] mice were a kind gift from Michael Lisanti (Jefferson University, PA).

BMMs were seeded onto tissue culture plates and allowed to adhere overnight in co-culture media (αMEM; 10% FBS; 3×10^2 U/ml CSF-1). The next day, media was either replaced with fresh co-culture media (BMM sample) or with sterile-filtered (0.22 μm) co-culture media conditioned with Met-1 cells for 24 hours (conditioned media sample). Met-1 cells were also cultured separately (Met-1 sample). Supernatants were collected and sterile-filtered at 6 hours, 12 hours, and 24 hours then assayed by enzyme linked immunosorbent assay (ELISA) for VEGF and chemokine (C-C motif) ligand 3 (CCL3; R&D Systems, Minneapolis, MN) per manufacturer's protocol. RNA was also isolated from all samples, extracted as described above and used for qrtPCR.

Results

TAMs Can Be Identified and Sorted by Flow Cytometry

In this study, TAMs were isolated from late-carcinoma stage primary tumors of the transgenic polyoma middle T oncoprotein mouse model of breast cancer (PyMT)[25] crossed to the MacGreen animal in which EGFP is expressed under control of the *Csf1r* promoter to identify myeloid-lineage cells.[26] Recently, it has been reported that mouse neutrophilic granulocytes express *Csf1r* mRNA and are EGFP[+] in the MacGreen animal.[40] To determine whether this was true in the tumor microenvironment, flow cytometric analysis was performed on EGFP^{+} cells, and compared with unstained controls. Results indicate that these cells comprise a heterogeneous population bearing mixed expression of the cell-surface macrophage-specific markers, CSF-1R and F4/80 and

granulocyte-specific marker, Gr1 (Figure 1A, panels i, ii, and iv). In contrast, all cells expressed the myeloid-specific marker, CD11b (Figure 1A, panel iii).

Previous work has indicated that the ability of macrophages to phagocytose fluorochrome-conjugated dextran can be exploited for the identification of TAMs in tumor-bearing animals and that a population of these dextran[+] TAMs promote carcinoma cell invasion.[18] To determine whether the dextran[+]/EGFP^{+} cells comprised a pure population of TAM, MacGreen tumor-bearing animals were tail-vein injected with Texas-Red conjugated 70KD dextran two hours before sacrifice for further flow cytometric analysis. Single-cell suspensions were mechanically prepared on ice and then stained with the macrophage-specific antibody, F4/80 and the granulocyte-specific antibody Gr1. Results indicate that while EGFP^{+} cells from the MacGreen tumor were either F4/80[+] or Gr1^{+}, dextran[+]/EGFP[+] double-positive cells were enriched for F4/80 and not Gr1 positivity (Figure 1B). To determine identity, dextran[+]/EGFP[+] and dextran[−]/EGFP^{+} cells were sorted by flow cytometry for cytospin analysis. Whereas cells exhibiting polymorphonuclei (typical of neutrophils) were abundant in dextran[−]/EGFP^{+} cells together with mononuclear cells, none were identified in the dextran[+] population (Figure 1C).

F4/80[+] TAMs Have a Distinct Distribution Compared with Gr1^{+} Cells

Flow cytometry results indicate F4/80[+] and Gr1^{+} cells in the tumor microenvironment differ in capacity to phagocytose dextran. Histologically, subpopulations of F4/80[+] TAMs are visible in the PyMT animal.[11,41] These subpopulations include TAM localized in stromal, hypoxic, perivascular, and perinecrotic areas of late-stage tumors. To determine how the distribution of Gr1^{+} cells compared with TAMs, serial frozen sections from late stage
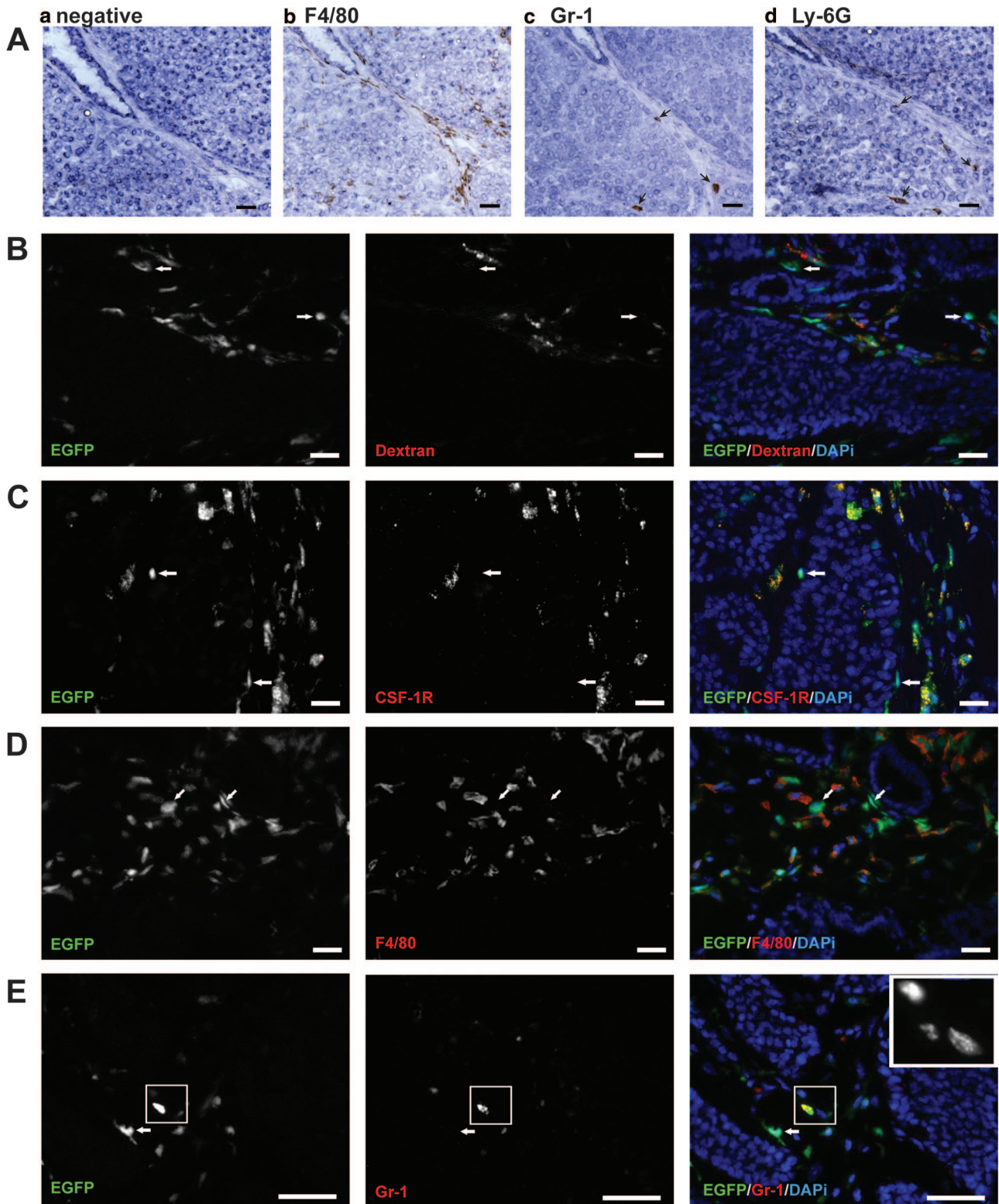
Figure 2. Localization of TAMs in PyMT Tumors. **A:** Immunohistochemistry was performed on serial frozen sections of late stage PyMT tumors. Negative irrelevant control antibody (**a**). F4/80 staining illustrates localization of macrophages in the stroma and diffusely in the tumor islands (**b**). Gr-1 staining is more sparse and restricted to stroma/peri-vascular areas of the tumor (**c**). Ly-6G staining is also restricted to stroma/peri-vascular areas of the tumor and appears to overlap with Gr-1 staining as indicated by **black arrows** (**d**). Scale bar = 50 μm. **B:** Localization of dextran labeling (red) cells within MacGreen tumor. **C:** Localization of CSF-1R (CD115) cells within MacGreen tumor. **D:** Localization of F4/80 cells within MacGreen tumor. **E:** Localization of Gr1 cells within MacGreen tumor. **Inset** ×2 magnification of DAPI-stained nuclei in merged image. **B left panels** green (EGFP) channel, **middle panels** red channel, **right panels** merged image with DAPI. **Arrows** indicate cells that are EGFP[+], but do not express cell-type specified markers. Scale bars = 10 μm.

tumors were stained with both antibodies. As previously reported, F4/80^{+} cells were broadly distributed[12,18] (Figure 2A, panel i, ii). In contrast Gr1^{+} cells were not as broadly distributed and appeared to be primarily restricted to perivascular and stromal areas of the late stage tumor (Figure 2A, panel iii). The Gr1 antibody reacts with both Ly-6C and Ly-6G protein. While Ly-6C is present on cells of the mononuclear lineage,[42] Ly-6G is expressed predominantly on polymorphonuclear neutrophils.[40] Consistent with the flow cytometry and cytospin data, staining for Ly-6G on late-stage tumor overlaps with staining for Gr1 in serial sections indicating further that these Gr1+ cells are distinct from the F4/80[+] TAMs sorted for array studies (Figure 2A, panel iv).

Immunofluorescence was performed on late stage MacGreen tumors to examine the co-localization of EGFP with mature macrophage markers including pre-injected dextran (Figure 2B), CSF-1R external epitope (Figure 2C) and F4/80 (Figure 2D). Arrows indicate examples in each panel where GFP expression fails to co-localize with the queried macrophage marker. Consistent with flow-cytometry the granulocyte marker, numerous EGFP^{+} cells co-localized with the granulocyte marker, Gr1. Additionally, GFP[+]/Gr1^{+} cells appear to have polymorphonuclei consistent with the cytospin data and the identification of these cells as neutrophils (Figure 2E and inset). All these studies taken together indicate that the MacGreen mouse

labels neutrophils in tumor stroma, and that they are abundant. However, this work also validates the use of a marker, such as dextran, that is able to distinguish between F4/80[+] and Gr1^{+} cells in our model system. Henceforth in this study, TAMs are identified as dextran^{+}; *Csf1r*-EGFP^{+}; F4/80^{+}; Gr1^{-}; Ly-6G^{-}; CD11b^{+} cells.

TAMs Exhibit a Unique Gene Expression Profile Compared with Splenic Macrophages

Using *EGFP* expression from the *Csf1r* promoter and Texas Red conjugated dextran as criteria for macrophage selection, TAMs were sorted from PyMT late stage tumor and splenic macrophages with the same phenotype were sorted from non-transgene bearing littermate controls. All isolation steps were maintained at 4°C. RNA was extracted from sorted populations and then validated for quantity and quality before being linearly amplified to yield sufficient material for array hybridization. Four biological repeats were hybridized for each group (Figure 3A).

Hybridizations were normalized using the Robust Multichip Average method[28] as described in the "Materials and Methods" and log averages of normalized expression values for TAMs were plotted against values for splenic macrophages (Figure 3B). The strong correlation ($r = 0.9698$) between the two groups indicates overall
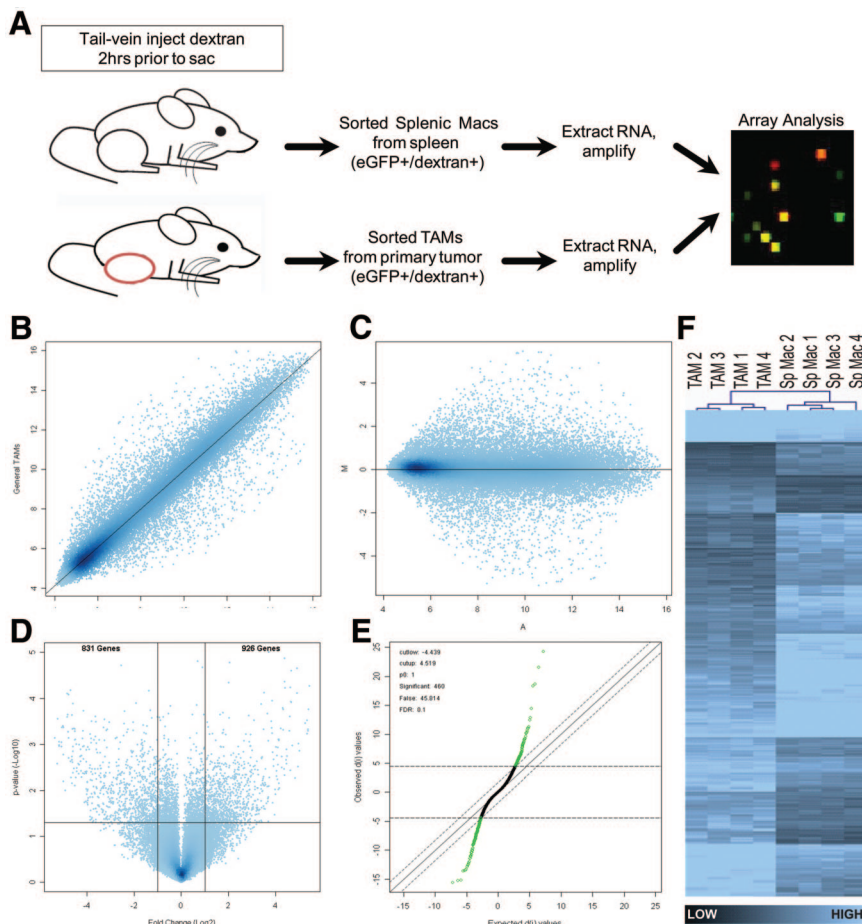

Figure 3. TAM gene expression can be robustly assayed by high-density oligoarrays and exhibit a unique expression signature compared with tissue splenic macrophages. **A:** Schematic of the experimental procedure to compare TAMs versus splenic macrophages. **B:** Scatter plot. Average log expression of each queried transcript for TAMs compared with splenic macrophages with linear regression line overlaid ($r = 0.9698$). **C:** MA-plot. Log ratio of Cy3 expression (TAM) and Cy5 expression (splenic macrophages) (M), plotted against average of the log intensities (A). Symmetry with respect to horizontal line indicates lack of dye bias in array. **D:** Volcano plot. Log fold change between groups versus significance level as calculated by *t*-test ($-\log_{10}[P$ value]). Horizontal line marks P value = 0.05. Vertical lines mark log fold change less than or greater than 1. With these criteria, 926 genes are called as increased in TAMs compared with splenic macrophages, and 831 genes are called as decreased. **E:** SAM plot. Significance Analysis of Microarrays with delta value of 1.74, false discovery rate of 0.1. Called genes: 210 increased expression in TAMs; 242 decreased expression; 8 not regulated by fold-change criteria. **F:** Dendogram and hierarchical clustering. Genes depicted are those called by SAM analysis. Dark shaded boxes indicate low expression; light shaded boxes indicate higher expression. Clustering analysis robustly separate the TAM samples from splenic macrophage samples.

successful processing. No dye-bias was present as indicated by the MA plot (Figure 3C), which plots the log ratio (M) of the two dyes used in the hybridization, Cy3 and Cy5, to the average of the log intensities (A).

A 'volcano plot' was generated to graphically represent transcripts of increased ($\log_2$ ratio greater than 1) or decreased ($\log_2$ ratio less than -1) abundance in TAMs as compared with splenic macrophages (Figure 3D). Vertical lines demarcate these fold-change boundaries. For each transcript, a student's *t*-test was performed between the TAM and splenic macrophages. These data are plotted on the $-\log_{10}$ transformed *y* axis. Transcripts with a *P* value less than 0.05 (indicated by horizontal line on the transformed axis at 1.36) were considered statistically significant. Thus, those transcripts in the upper left-hand panel (831 genes) were significantly of reduced abundance in TAMs as compared with splenic macrophages and those transcripts in the upper right-hand panel (926 genes) were of increased abundance.

While volcano plots are useful for visualizing data, in the setting of high-density expression arrays, caution needs to

be exercised when applying a generally acceptable significance value to >35,000 sets of data. The SAM method[31] compares variance among all probes to the variance of each individual probe to better predict significance on a high-density platform. A user-defined delta value establishes cut-offs for transcripts significantly regulated as compared with the overall population. For the arrays in this study, a delta of 1.74 was used (Figure 3E). With this delta value, 460 significant transcripts were called with a 10% false discovery rate. For high-density expression arrays, SAM is a more rigorous and stringent gene selection tool as compared with *t*-tests and therefore the transcript abundance of 460 regulated genes generated from this method is used for further analysis (see Supplemental Table S1 available at *http://ajp. amjpathol.org*).

Figure 3F illustrates hierarchical clustering of these 460 transcripts called by SAM analysis. Notably, all four TAM samples cluster together as did the splenic samples. This suggests that TAM exhibit a unique gene expression profile compared with splenic macrophages. Using the less stringent gene selection criteria (ie, *P* <
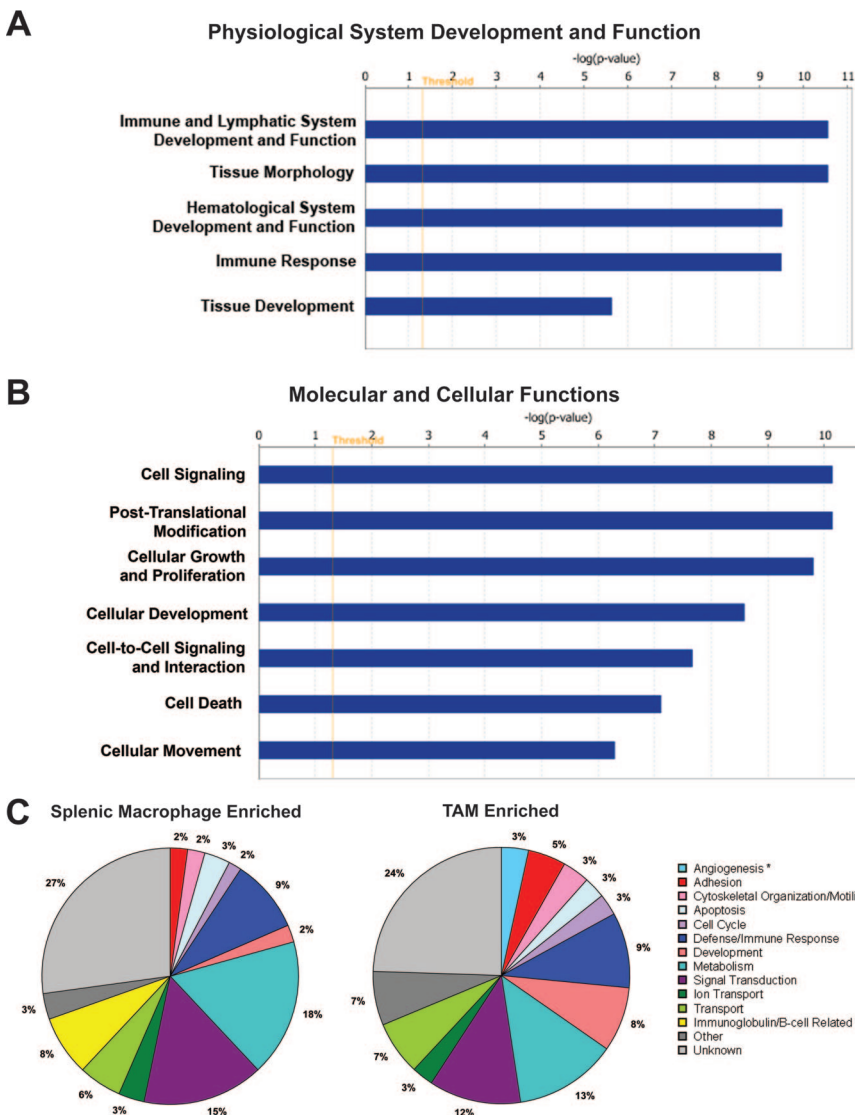
A

B

Figure 4. Transcripts mediating immune function and tissue development are differentially regulated in TAMs. Genes selected by SAM subjected to bioinformatics analysis using IPA. **A:** Most significantly enriched functional groups relating to physiological system development and function. **B:** Most significantly enriched groups pertaining to molecular and cellular function. Both panels include corresponding *P* values calculated through IPA using right-tailed Fisher Exact Test. **C:** Primary gene ontological classification was designated for each differentially regulated gene and a pie-chart was created to visualize enriched gene ontologies in transcripts decreased in abundance (**left panel**) and increased abundance (**right panel**) in TAM. Legend on right ascribes classifications. Percentages associated with each wedge represent percent enrichment in regulated gene population.

C

0.05), the TAM samples remain clustering separately from splenic macrophages (data not shown).

TAMs Express Increased Transcript Abundance of Genes Regulating Immune Response, Developmental Processes and Angiogenesis

Differentially expressed transcripts were uploaded to the IPA database for exploration of enriched biological functions in TAMs (Figure 4A). As previously reported, with a high significance ($P = 3.2 \times 10^{-10}$) the 'immune response' is differentially regulated between TAMs and splenic macrophages. Additionally physiological processes relating to 'tissue development' were significantly regulated ($P = 2.3 \times 10^{-6}$). Subcategories of tissue development include 'adhesion' and 'angiogenesis' and were also individually regulated with high significance ($P = 2.3 \times 10^{-6}$ and $P = 3.6 \times 10^{-3}$, respectively). A similar analysis was performed on the specific molecular and cellular functions differentially regulated. Processes such as 'cell signaling' and 'posttranslational modification' were most significantly differentially expressed while processes such as 'cell-to-cell signaling' and 'cellular movement' were also enriched in the pooled abundance of differentially regulated transcripts (Figure 4B).

To gain a better appreciation for functional processes regulated in TAMs, the biological process gene ontological classification for each transcript was determined through the use of tools such as the Gene Ontology Project and the DAVID, in addition to extensive literature review. After establishing an ontological designation for each differentially regulated transcript, two pie charts were created to compare the results of enriched functions in the up-regulated group (increased transcript abundance in TAMs) to those in the down-regulated group (decreased transcript abundance in TAMs) (Figure 4C). As suggested by the analysis performed on Ingenuity, genes related to immune/defense response were abundantly regulated, as were genes involved with developmen-

tal processes. Interestingly, while 'angiogenesis' was a significantly regulated process as indicated through IPA when analyzing the entire called 460-gene data set, pie chart analysis indicates that angiogenesis-related genes were uniformly of increased abundance in TAMs. Re-analyzing the up- and down-regulated genes by IPA separately confirms this finding with the P value for 'angiogenesis' becoming more significant in the up-regulated group ($P = 2.6 \times 10^{-4}$) and not significant in the down-regulated group.

In the decreased group, a number of immunoglobulin and B-cell specific transcripts are called. This most likely relates to a primary function of splenic macrophages—engulfment and disposal of apoptotic leukocytes and may also explain the number of apoptosis-related genes regulated in this group.[43] Considering the proportion of apoptosis-related transcripts in the up-regulated group this may suggest that TAMs in the late stage tumor maintain the ability to recognize and engulf apoptotic tumor cells thus retaining a key function of innate immunity. This is consistent with the selection of dextran phagocytosis as a marker of macrophages in this analysis.

Taken together, the results from the bioinformatic processing of regulated transcripts in TAM versus splenic macrophages indicates that TAMs differentially regulate groups of genes mediating immune response, tissue developmental processes and angiogenesis, in addition to other physiologically relevant processes. These data suggest a functional profile of TAMs as uniquely poised to perform tissue trophic functions while at the same time maintaining certain key roles in regulating innate immunology.

Quantitative Real-Time PCR Validates the Gene Expression Array Data from Numerous Ontological Clusters

Numerous genes identified through the microarrays were validated by quantitative real-time PCR (qrtPCR) using rigorously designed and tested primers. Samples used for qrtPCR were biological replicates to further validate the
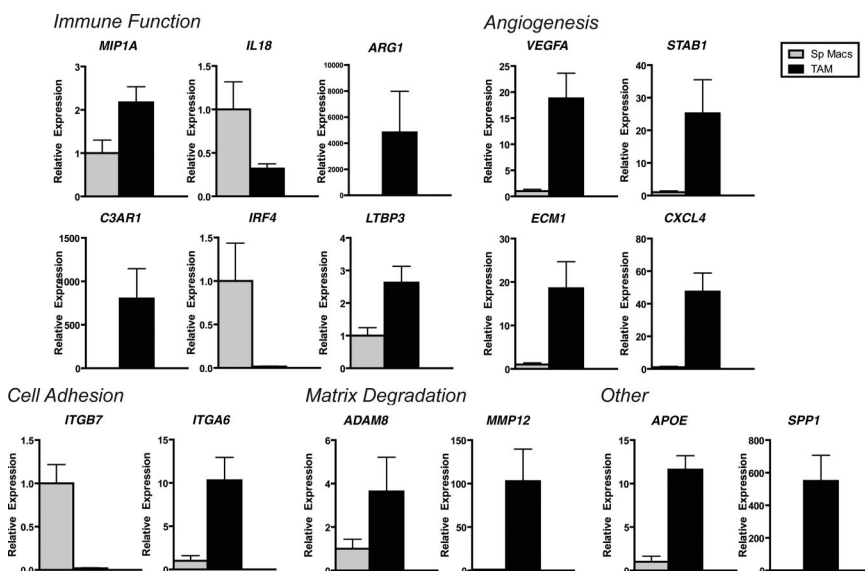
Figure 5. Quantitative rtPCR validates gene expression array data from numerous ontological clusters. Sixteen genes identified as differentially regulated from arrays validated using qrtPCR using separate biological repeats. All samples were normalized to the housekeeping gene, *Pp1a*. Data shown indicates relative expression of TAMs (black bars) with respect to splenic macrophages (gray bars), set to one. Gene symbol abbreviations listed in Table 1.

array data with independent repeats. While for array analysis, it was necessary to use mRNA that had undergone two rounds of amplification to obtain a sufficient quantity of material, it was found that one round of amplification was adequate to amplify sufficient material for qrtPCR. The relative expression of each gene for each sample was calculated with respect to the housekeeping gene, *Pp1a*. TAM samples were normalized to the expression levels in splenic macrophages (mean set at 1) and relative expressions of TAMs compared with splenic macrophages for each gene are indicated. All genes tested validated results from the array. Results for sixteen genes up- and down-regulated are shown in Figure 5. To verify bioinformatic results, analyzed genes were selected from various ontological clusters and arranged accordingly. As the array data suggests, genes regulating immune function, angiogenesis, cell adhesion, and matrix degradation are transcriptionally regulated.

TAMs Secrete Numerous Factors Regulating Tumor Angiogenesis

It has been established that TAMs have a significant role in regulating tumor angiogenesis[27,44,45] at least in part through their ability to secrete VEGF.[12] With the gene expression array results, it was striking to note that a number of other differentially regulated transcripts that are ontologically called to have a role in regulating tumor angiogenesis were also identified. Using IPA, the cellular localization of all differentially regulated transcripts called to have a role in angiogenesis was determined (Figure 6A). Most of the related proteins are localized to the extracellular space suggesting the angiogenic regulatory role that TAMs play in the tumor microenvironment. The log ratio was superimposed atop the data and illustrates that of 13 of 14 differentially regulated genes ($P < 0.05$)
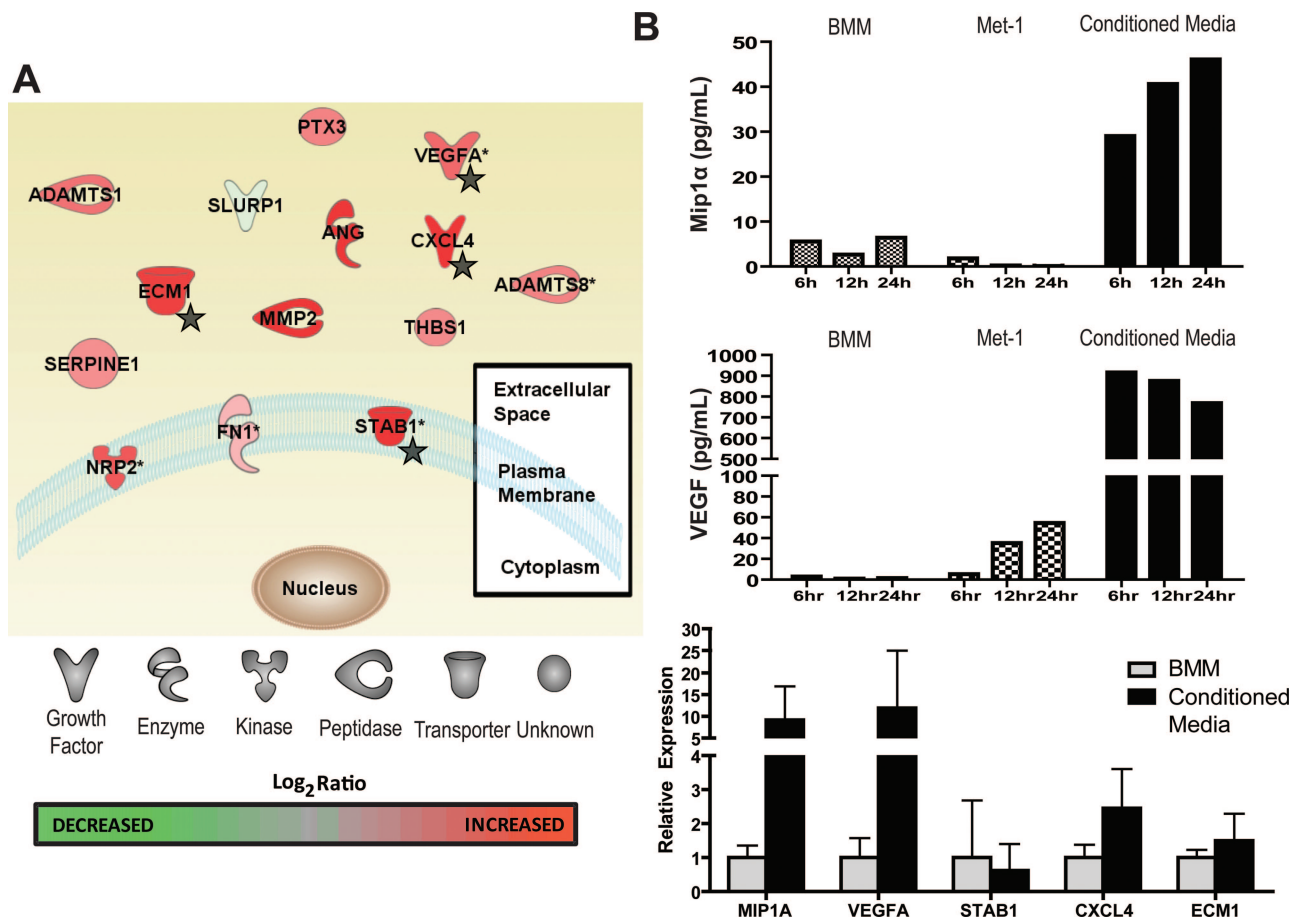

Figure 6. TAMs regulate tumor angiogenesis through multiple mediators. **A:** Differentially regulated transcripts ($P < 0.05$) that were ascribed a role in angiogenesis (Gene Ontology/IPA) were plotted on a schematic indicating cellular localization. Log ratio data were overlaid to indicate those genes of increased abundance in TAMs (red) and those of decreased abundance (green). Stars label those results validated by qrtPCR. To determine whether these transcripts can be regulated in macrophages by tumor cells, *in vitro* cultures were established between primary BMMs and Met-1 cells. **B:** ELISA for Mip1α (**top**) and VEGF (**middle**) is used to compare the validity of the *in vitro* system to results from isolated TAMs. BMMs were cultured alone and treated with Met-1 cell conditioned media. Shown are representative results from three experiments of collected supernatants at 6, 12, and 24 hours. (**bottom**) qrtPCR at 12 hours for BMMs treated with Met-1 conditioned media and control BMMs. Results standardized to *Pp1a* housekeeping gene. Relative expression is normalized to that of the control BMM. Abbreviations: ADAMTS 1/8 (ADAM metallopeptidase with thrombospondin type 1 motif, 1/8); ANG (angiogenin); CXCL4 (platelet factor 4 (chemokine (C-X-C motif) ligand 4); ECM1 (extracellular matrix protein 1); FN1 (fibronectin 1); MIP1A (chemokine (CC motif) ligand 3); MMP2 (matrix metallopeptidase 2); NRP2 (neuropilin 2); PTX3 (pentraxin-related gene); SERPINE1 (serpin peptidase inhibitor, clade E, member 1); STAB1 (stabilin 1); SLURP1 (secreted LY6/PLAUR domain containing 1); VEGFA (vascular endothelial growth factor A).

relating to angiogenesis are of increased transcript abundance in TAMs compared with splenic macrophages.

One cellular function particularly enriched in TAMs was cell-to-cell interaction. To ask the question of whether the increased abundance of angiogenic-related transcripts was related to interactions between the macrophages and carcinoma cell, cultures were set up such that BMMs were plated overnight and then overlaid with fresh media or media conditioned by Met-1 carcinoma cells for 24 hours.

ELISAs for VEGF and CCL3/Mip1α were performed on collected supernatants as a read-out for the validity of the *in vitro* cultures. Both molecules emerged as an up-regulated chemokine on the gene expression arrays as well as by qrtPCR. Increasing amounts of Mip1α protein was found over time secreted into the supernatants of BMM's treated with Met-1 conditioned media. Negligible amounts of Mip1α were secreted by BMMs or Met-1 cells alone (Figure 6B i). Similar results were seen with VEGF, although the carcinoma cells alone exhibited higher levels of basal secretion than BMMs (Figure 5B ii). The results for VEGF secretion are consistent with co-cultures between an ovarian tumor cell line and differentiated peripheral blood mononuclear cells as previously reported[46] suggesting

conservation between tumor cell and macrophage types used.

At 12 hours after introduction of conditioned medium, RNA was isolated from BMMs treated with Met-1 cell-conditioned media and control BMMs. Quantitative real time PCR indicated that consistent with ELISA data, Mip1α, and VEGFA was increased in BMMs treated with conditioned media. In addition, chemokine (C-X-C motif) ligand 4 (CXCL4), another molecule shown to regulate tumor angiogenesis, was increased in BMMs treated with conditioned media. Stabilin 1 (Stab1) and extracellular matrix protein 1 (ECM1) two other molecules identified in the array analysis were not significantly up-regulated by the tumor cell derived conditioned media (Figure 5b iii).

TAM Gene Expression Signature Significantly Overlaps with Enriched Transcripts in Fetal Macrophages

As indicated in Figure 4, the TAM gene expression signature is enriched for molecules affecting tissue morphology and development. To further assess the significance of this point, significantly regulated TAM transcripts ($P <$
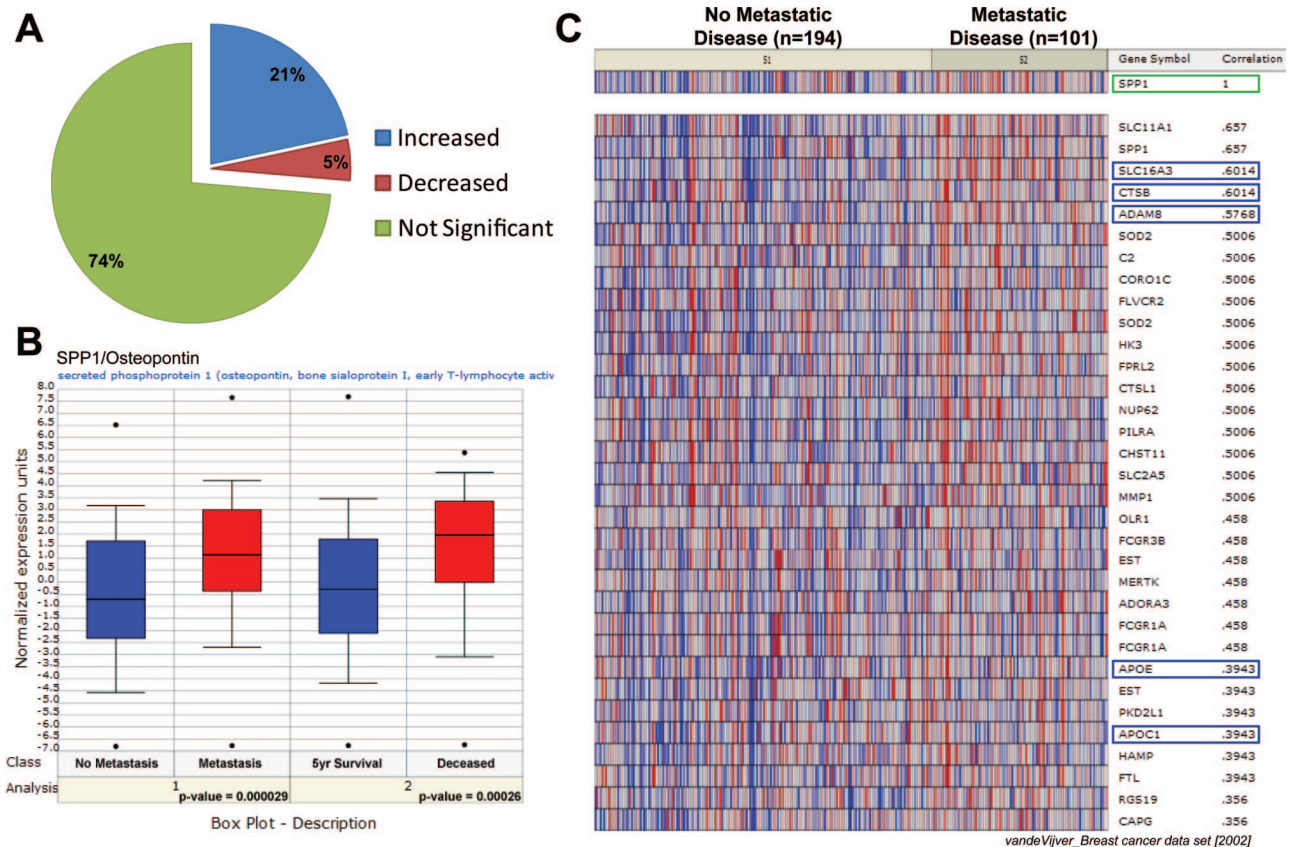

Figure 7. Murine TAM signature overlaps with murine fetal macrophages and human breast cancer. **A:** Comparison of TAM differentially regulated transcripts ($P < 0.05$) and top enriched transcripts identified in fetal macrophages[35] indicates that the two macrophage populations are associated ($P < 0.001$, Fisher's Exact Test). Overlapping transcripts between both groups and increased in TAMs are in blue and identified in Table 2. Overlapping transcripts decreased in TAMs are in red and identified in Table 3. **B:** Oncomine-generated box-and-whiskers plots for Spp1/Osteopontin expression considering metastasis status (**left**) and 5-year survival (**right**) using vandeVijver_Breast 2002 breast cancer gene expression dataset. *P* values generated using student's *t*-test through Oncomine. **C:** Oncomine-generated heat map from vandeVijver_Breast dataset. **Left** (S1) are from individuals with no evidence of metastatic disease and **right** (S2) with metastasis. Top correlated transcripts to Spp1 expression are ranked on the right with associated coefficients. Boxed molecules were also identified as differentially expressed between TAMs and splenic macrophages. All symbols are official gene symbols.

Table 2. Enriched in Embryonic Phagocytes; Increased in TAMs

Symbol	Gene name	Log$_2$ ratio	P value
Msr2	Macrophage scavenger receptor 2	4.9161	2.22×10^{-5}
Ccl12	Chemokine (C-C motif) ligand 12	4.1604	0.007088
Ccl2	Chemokine (C-C motif) ligand 2	4.054525	0.003846
C3ar1	Complement component 3a receptor 1	3.654725	0.000217
Ltc4s	Leukotriene C4 synthase	3.6327	0.019116
Abca1	ATP-binding cassette, subfamily A (Abc1), member 1	3.561275	0.007704
Ccl7	Chemokine (C-C motif) ligand 7	3.42445	0.02399
Npl	*N*-acetylneuraminate pyruvate lyase	3.407825	0.017164
Ms4a7	Membrane-spanning 4-domains, subfamily A, member 7	3.4012	0.007773
Gas6	Growth arrest specific 6	3.225975	0.033465
Cyr61	Cysteine rich protein 61	3.21085	0.024879
Hexb	Hexosaminidase B	3.109725	0.016917
Spp1/Opn	Secreted phosphoprotein 1 (osteopontin)	3.08975	0.000306
Apoe	Apolipoprotein E	2.9758	0.014086
Gatm	Glycine amidinotransferase	2.867175	0.025479
Anxa3	Annexin A3	2.6821	0.020122
Lgals9	Lectin, galactose binding, soluble 9	2.445575	0.001631
P2ry6	Pyrimidinergic receptor P2y, G-protein coupled, 6	2.43605	0.001127
Ccrl2	Chemokine (C-C motif) receptor-like 2	2.387875	0.016938
Adfp	Adipose differentiation related protein	2.103775	0.012731
aMs4a6d	Membrane-spanning 4-domains, subfamily A, member 6d	2.0592	0.038571
Fcgr1	Fc receptor, IgG, high affinity I	2.03215	0.019295
Gp49b	Glycoprotein 49b	1.701525	0.008324
Ehd4	Eh-domain containing 4	1.68445	0.004299
Ier3	Immediate early response 3	1.61345	0.020651
Pros1	Protein S (alpha)	1.59845	0.017915
Ccr1	Chemokine (C-C motif) receptor 1	1.540675	0.039695
Itgam	Integrin alpha M	1.135375	0.003645
Adcy7	Adenylate cyclase 7	1.04645	0.026588
Rgs1	Regulator of G-protein signaling 1	1.019575	0.020009
Dusp1	Dual specificity phosphatase 1	0.867275	0.044837
Sat1	Spermidine/spermine N1-acetyl transferase 1	0.784975	0.027386

0.05) were compared with the previously published gene expression signature of macrophages isolated at day 15.5 postconception mouse embryos.[34] Of the top 150 transcripts most enriched in fetal macrophages,[35] 26% of molecules were also differentially regulated in TAMs (Figure 7A). Thirty-two overlapping molecules were increased (Table 2) and 7 were decreased (Table 3) in abundance in TAMs. Results indicate that there is a positive association between both macrophage populations (Fisher's Exact Test, $P < 0.001$).

Osteopontin Expression Correlates with Multiple TAM-Expressed Transcripts in Human Breast Cancer

Osteopontin, or secreted phosphoprotein 1 (Spp1), is a phosphorylated integrin-binding glycoprotein and one example of a molecule enriched in fetal macrophages

and increased in TAMs (Figure 5 and Table 2). Spp1 is known to be expressed by macrophages,[47,48] including TAMs.[49] Moreover, Spp1 expression correlates with disease prognosis for numerous cancers including breast cancer where it is associated with disease progression and metastasis (reviewed[50,51]). This is evinced using the Oncomine cancer microarray database[36,37] to mine a breast cancer gene expression study of 295 human breast carcinoma biopsies (vandevijver_Breast)[52] indicating increased expression of Spp1 correlates with increased coincident tumor metastasis and decreased 5-year survival (Figure 7B).

Spp1's previous association with macrophage activity and tumor progression, as well as access to the whole genome, validated and pure TAM gene expression array data reported here, rationalized our use of Oncomine to determine transcripts co-expressed with Spp1 in the vandevijver_Breast dataset. Using Oncomine, ranking of

Table 3. Enriched in Embryonic Phagocytes; Decreased in TAMs

Symbol	Gene name	Log$_2$ ratio	P value
Igh-6	Immunoglobulin heavy chain 6 (heavy chain of IgM)	−3.985425	0.006788
Fcna	Ficolin A	−3.628125	0.049599
Cd163	Cd163 Antigen	−2.73325	0.002958
Mgl1	Macrophage galactose *N*-acetyl-galactosamine specific lectin 1	−1.91685	0.049294
S100A9	S100 calcium binding protein A9 (calgranulin B)	−1.7435	0.04624
Ier2	Immediate early response 2	−1.122525	0.001927
Amh	Anti-mullerian hormone	−0.4795	0.029225

the most correlated transcripts revealed multiple molecules that are co-expressed with Spp1 in malignant human breast tumors and also differentially expressed in our TAMs, isolated from transgenic murine mammary tumors (Figure 7C). *Adam8* and *Apoe*, specifically are two identified differentially regulated transcripts in TAMs that were confirmed by qrtPCR (Figure 5) and found to be co-expressed with *SPP1* in the human study. Three other molecules, solute carrier family 16, member a3 (*Slc16a3*), cathepsin b (*Catb*) and apolipoprotein c1 (*Apoc1*) were also differentially regulated between TAMs and splenic macrophages (*P* < 0.05) and co-expressed with the human ortholog of *SPP1*.

Discussion

In experimental models of breast cancer, TAMs are important components of the microenvironment that promote tumor progression and enhance metastatic potential. These activities contrast with the classical immunological perspective, that they are important components of the immune system due to their ability to engulf and destroy harmful microbes, as well as for their ability to present antigens to T-cells to initiate a more professional assault on disease pathogens. This latter view would suggest that macrophages would play an important role in the rejection of tumors that were perceived as foreign. Therefore, more insight into how macrophages are not simply permissive, but facultative of tumor growth in the tumor microenvironment has the potential to shed insight into cancer and macrophage biology and may expose new therapeutic opportunities. In the experiments described in this paper we used high density oligonucleotide arrays to dissect putative mediators of these trophic macrophage functions in the tumor microenvironment.

Previously, experimental studies have demonstrated functions for TAMs that have provided insight into how macrophages facilitate tumor progression. TAMs have been shown to regulate tumor angiogenesis,[12,53,54] promote carcinoma cell motility and intravasation,[18,55–57] promote matrix degradation,[16,58] and modify the inflammatory context of the tumor microenvironment.[22,59] Many of these tissue trophic functions of TAMs are believed to recapitulate functions of macrophages during development. For example, a recent observation has been the extent to which macrophages direct terminal end bud morphogenesis in the mouse developing mammary gland through promotion of collagen fibrillogenesis.[20,21,60,61] It is hypothesized that similar to their role in promoting "invasion" of the terminal end bud into the surrounding mammary fat pad, TAMs promote invasion of the leading edge of a late-stage carcinoma during metastasis.[15] While these mechanisms have been well documented, the molecular basis of their control remains obscure, in particular how TAMs can be directed away from being immunologically potent cells to those that potentiate tumor progression to malignancy. In addition, growing evidence of the contributions of other bone marrow cells to tumor progression including myeloid derived suppressor cells,[62] mesenchymal stem cells,[63] VEGFR1+ hematopoietic progenitors[64] and CCR1+ myeloid cells[65] underscores the necessity for rigorous identification of cell population before initiating gene expression studies, as demonstrated here.

In this context the macrophage biology field has recently benefited from a number of gene expression microarray studies that have defined transcriptomes of these cells from a range of tissue microenvironments. To note, prudence is required during the analysis and interpretation of any gene expression studies as results are dependent on pure samples isolated under conditions that minimize technically introduced gene expression changes. This is especially true for macrophages that readily adapt and react to new environments such as during a common method of isolation that involves adhesion to plastic surfaces.[22] To overcome these problems in this study we used flow cytometry to isolate transgenically (EGFP) labeled macrophages directed by the *Csf1r* promoter from tissue maintained at 4°C that has not been enzymatically processed nor requires adhesion steps for purification. After an early observation that these MacGreen animals have at least two populations of GFP-labeled myeloid cells ([40] and Figure 1), it was recognized that another marker was needed to identify macrophages. It has been previously described in the lab that the use of fluorochrome-labeled 70-kDa dextran could be use to exploit the pathognomonic phagocytic nature of macrophages.[27] Considering the leakiness of immature neovessels in the tumor microenvironment that are resistant to complete clearing via PBS perfusion through the right ventricle perimortem, using dextran to identify TAMs also excludes monocytes from the sorted population. Thus these steps ensure the purification of a true macrophage population with minimal perturbation and we describe a pure population of tissue macrophages that are F4/80+, CD11b+ and importantly, Gr1−, thus fulfilling attributes of a mature macrophage population.

TAMs as defined above were isolated from the transgenic PyMT mouse and an equivalent population of splenic macrophages from littermate controls were isolated and analyzed by high-density oligoarrays. Splenic macrophages were chosen as a reference tissue macrophage population due to the feasibility of collecting large numbers of cells using a similar protocol to that for the TAM isolation and their defined immunological functions. We chose not to use spleen from the same tumor-bearing mice since primary tumors are known to affect the myeloid populations in spleen. Particularly there is recruitment of myeloid cell suppressor cells that express the Cd11b marker.[66,67] However, these cells also express Gr1 and are mostly granulocytic precursors[66,67] and thus would have been excluded by our selection criteria. Nevertheless we wished to use an unperturbed population that was in a resting state with selection for identical markers as the TAMs. This rigorous selection also overcomes much of the problem of splenic red and white pulp macrophage heterogeneity and the abundance of dendritic cells in this organ. These cells have different immunological responses and thus we chose mice that had not been immunologically challenged.[68,69] In future studies it

will be interesting to compare the resting splenic macrophages with a similar population from tumor bearing mice. In this study, hierarchical clustering demonstrated that the two macrophage populations cluster separately. This indicates that TAMs and splenic macrophages comprise separate populations based on gene expression. Using the stringent SAM analysis, 460 transcripts were identified as differentially regulated among the two populations (see supplemental Table S1 available at *http://ajp.amjpathol.org*). All results tested by qrtPCR in subsequent biological repeats validated the array.

Bioinformatic analysis of the microarray results indicates that pro-tumor functions previously shown to be characteristic of TAMs, are transcriptionally regulated compared with splenic macrophages suggesting that they are directed within the tumor microenvironment. These include a number of differentially regulated transcripts that could encode angiogenic molecules (Figure 4 and supplemental Table S1 available at *http://ajp.amjpathol.org*). There is a similar enrichment identified for transcripts known to be involved in cellular and tissue developmental processes that are of increased abundance in TAMs. Furthermore, comparison of the data put forth here to previous expression arrays of macrophages in the developing embryo indicate several genes that are expressed (such as *Spp1, Apoe, C3ar1, Trem2,* and *Msr2*) and differentially regulated in TAMs,[34] Figure 5 and supplemental Table S1 available at *http://ajp.amjpathol.org*). Previous TAM expression array studies using 10K cDNA microarrays[22] and cytokine bead arrays[23] were used to specifically examine regulated genes of immunological significance. Numerous immune-related transcripts were similarly regulated in this study, for example, *Tgfb, C3ar1, Cxcl16, Msr2,* and *Il18*. The recognition that much of this previous data derived from different xenograft tumor models aligns here, with TAMs derived from transgenic induced spontaneous mammary tumors indicates the extent of conservation of TAM activities between tumor types and further suggests the extent of therapeutic benefit by targeting specific TAM molecules.

While overall, the immunological suppressive phenotype of TAM is exhibited as previously ascribed to the so-called M2 macrophages,[70] in at least one case in TAMs isolated from the PyMT animal a pro-inflammatory chemokine, CCL3 (Mip1α), was identified as being increased in TAMs in contrast to previously published data.[22] This fact was validated by qrtPCR and further by *in vitro* culture studies in which BMMs were treated with media conditioned by Met-1 cells, a tumor cell line derived from the PyMT mouse[39] by ELISA and qrtPCR. This suggests that in fact, some pro-inflammatory functions of TAMs in our model are maintained and illustrates a spectrum of activities in macrophages, as opposed to the dichotomous relationship suggested by the M1/M2 macrophage distinction. Indeed we would contend that the strictures of a binary description of macrophages as either M1 or M2 preclude the reality of a wide diversity of phenotypes displayed by macrophages in normal physiology and within the tumor microenvironment. Our description within

a well-defined TAM population of elements of both M1 and M2 phenotypes emphasizes this point.

To examine further the angiogenic phenotype of TAMs, pathway analysis using Ingenuity, was performed to identify those differentially regulated transcripts involved in angiogenesis. As indicated in Figure 4, using the stringent SAM criteria, angiogenesis-related transcripts are differentially regulated—however to better visualize the extent of this, pathway analysis was performed on those transcripts that had a significance of $P < 0.05$. Consistent with the SAM criteria, most angiogenesis-related transcripts differentially regulated were of increased abundance in TAMs using the less stringent criteria. Furthermore, most of these transcripts are known secreted proteins further indicating the role of TAMs in directing angiogenesis in the tumor microenvironment (shown in Figure 6A). The *in vitro* culture system described above was used to evaluate secretion of the most well-characterized pro-angiogenic cytokine, VEGF, and it was found that while the Met-1 carcinoma cells have basal secretion levels of VEGF, BMMs alone do not but can be stimulated to produce VEGF when treated with Met-1 conditioned media. Quantitative real time PCR showed a similar upregulation of another regulator of angiogenesis, CXCL4. In contrast, other angiogenic regulators such as Stab1 and ECM1 were not differentially regulated simply in the presence of Met-1 conditioned media suggesting that other cues, besides carcinoma cells, within the tumor microenvironment regulate these TAM phenotypes.

Because the bioinformatic analysis of differentially regulated TAM transcripts indicated a role for TAMs in tissue development, the array results were compared with the results of a recently published dataset for macrophages isolated from mouse embryos at day 15.5 postconception.[34] In support of the bioinformatics, there was significant overlap between the two populations suggesting conservation in function (Figure 7A). Ostepontin (Spp1) was further investigated because of its recognized function in macrophage activity[47,48] and tumor progression.[50,51,71] There has been discussion in the literature of the impact of macrophage-derived versus tumor-derived Spp1 on tumor progression spurred by a study of 154 lymph node negative breast cancer patients.[72] In this study, the authors found that while Spp1 was identified in infiltrating macrophages and lymphocytes in 70% of tumor biopsies, Spp1 was only identified in the carcinoma cells of 26% of tumor biopsies and this expression in tumor cells correlated with worse disease prognosis. No such association was identified in TAM expression. More recently, it was demonstrated that systemic tumor-cell derived Spp1 will alter the distribution of cells released from the bone marrow, and consequently better promote outgrowth of previously indolent tumors.[73] However, analysis of Spp1 against the van de Vijver breast cancer study demonstrated similar expression of several other differentially regulated TAM transcripts suggesting conserved function and source. Several of these transcripts including *Slc16a3, Ctsb, Adam8,* and *Apoe*, independently correlate with worse disease outcome in the van de Vijver study (data not shown) suggesting that similar

to tumor-derived Spp1, TAM-derived Spp1 also contributes to worse disease outcome.

These final results are significant because they once again demonstrate the validity of the transgenic PyMT murine model of breast cancer for modeling human disease. Even further, the results demonstrate that individual cell types can be studied in the context of a heterogeneous human tumor biopsy if a robust and validated gene expression signature from a pure cell population is available for comparison. While this point has previously been made in follicular lymphoma[74] and human breast tumors,[75] work herein provides a novel example of gaining insight into gene expression of human TAMs through work in a mouse model of breast cancer.

Taken together, this work illustrates that within the tumor microenvironment, macrophages display a gene expression signature capable of promoting tumor angiogenesis, preventing optimal immune surveillance and enhancing cellular processes supportive of tumor growth. Whereas this regulation is directed in part by the tumor cells, additional components of the tumor microenvironment (such as fibroblasts, adipocytes, and hypoxia) may also instruct macrophages to perform their functions within the tumor microenvironment. Considering the diversity of cell types within a given tumor, it is critical to rigorously identify and directly isolate well-characterized cell types to investigate their contribution to tumor growth. Using such an approach we have identified a tumor associated macrophage expression signature that will provide a valuable resource for further research into the role of these cells in human malignancy.

Acknowledgments

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