Induction of interferon signaling and allograft inflammatory factor 1 in macrophages in a mouse model of breast cancer metastases [version 1; peer review: awaiting peer review]

Wei Zheng¹,², Dejian Zhao³, Hui Zhang¹, Prameladevi Chinnasamy¹, Nicholas Sibinga¹, Jeffrey W. Pollard¹,⁴

¹Department of Developmental and Molecular Biology, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, New York, 10461, USA
²Division of Hematology and Oncology, Icahn School of Medicine at Mount Sinai, New York, New York, 10029, USA
³Yale Center for Genome Analysis, Yale University, New Haven, Connecticut, 06510, USA
⁴MRC Centre for Reproductive Health, Queen’s Medical Research Institute, The University of Edinburgh, Edinburgh, EH16 4TJ, UK

Abstract

Background: Metastatic breast cancer cells recruit macrophages (metastasis-associated macrophages, or MAMs) to facilitate their seeding, survival and outgrowth. However, a comprehensive understanding of the gene expression program in MAMs and how this program contributes to metastasis remain elusive.

Methods: We compared the transcriptomes of MAMs recruited to lung metastases and resident alveolar macrophages (RAMs) and identified a large variety of differentially expressed genes and their associated signaling pathways. Some of the changes were validated using qRT-PCR and immunofluorescence. To probe the functional relevance to metastatic growth, a gene-targeting mouse model of female mice in the C57BL/6J background was used to study allograft inflammatory factor 1 (AIF1, also known as ionized calcium-binding adapter molecule 1 or IBA1).

Results: Interferon signaling is one of the most activated pathways in MAMs, with strong upregulation of multiple components of the pathway and a significant enrichment for the gene signatures of interferon-alpha-treated human macrophages. *Aif1*, an interferon-responsive gene that regulates multiple macrophage activities, was robustly induced in MAMs. *Aif1* deficiency in MAMs, however, did not affect development of lung metastases, suggesting that AIF1 indicates MAM activation but is dispensable for regulating metastasis.

Conclusions: The drastically different gene expression profile of MAMs as compared to RAMs suggests an important role in promoting metastatic growth. Dissection of the underlying mechanisms and functional validation of potential targets in the profile may provide novel therapeutic strategies for the treatment of metastatic diseases.
Keywords
breast cancer, metastasis, macrophage, interferon, AIF1, IBA1, tumor microenvironment

Corresponding authors: Wei Zheng (wei.zheng@mssm.edu), Jeffrey W. Pollard (Jeff.Pollard@ed.ac.uk)

Author roles: Zheng W: Conceptualization, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; Zhao D: Data Curation, Formal Analysis, Writing – Original Draft Preparation; Zhang H: Investigation; Chinnasamy P: Investigation, Resources, Writing – Original Draft Preparation; Sibinga N: Resources, Writing – Original Draft Preparation; Pollard JW: Conceptualization, Funding Acquisition, Project Administration, Resources, Supervision, Visualization, Writing – Review & Editing

Competing interests: WZ had no competing interests. JWP had no competing interest when research was performed but is now a founder, director and consultant for "Macomics" an Immunoncology company. This is however, not in conflict with any of the research published here.

Grant information: This work was supported by the Wellcome Trust [101067], National Institutes of Health grant P30CA0133330, MRC Centre grant MR/N022556/1. P.C. and N.S. were supported by National Institutes of Health R01HL128066.

Copyright: © 2021 Zheng W et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Zheng W, Zhao D, Zhang H et al. Induction of interferon signaling and allograft inflammatory factor 1 in macrophages in a mouse model of breast cancer metastases [version 1; peer review: awaiting peer review] Wellcome Open Research 2021, 6:52 https://doi.org/10.12688/wellcomeopenres.16569.1

First published: 08 Mar 2021, 6:52 https://doi.org/10.12688/wellcomeopenres.16569.1
Abbreviations
AIF1: allograft inflammatory factor 1; BM: bone marrow; BMT: bone marrow transplantation; FACS: fluorescence-activated cell sorting; GSEA: gene set enrichment analysis; IFN: interferon; iv: intravenous; IPA: ingenuity pathway analysis; IVIS: in vivo imaging system; MAMs: metastasis-associated macrophages; RAMs: resident alveolar macrophages; RNA-seq: RNA-sequencing; WT: wild type.

Introduction
Tumor-associated macrophages are important in multiple steps of tumorigenesis and progression, including regulation of tumor cell invasion into the stroma, intravasation into blood vessels, seeding at distal organs, angiogenesis, inflammation and immune suppression (Qian & Pollard, 2010). Compared to the abundant knowledge of tumor-associated macrophages at primary sites, the role of the macrophages at metastatic sites, i.e. metastasis-associated macrophages (MAMs), has only begun to emerge in recent years. In mice, lung metastases resulting from mammary cancer have been shown to recruit MAMs that are CD11B⁺CD11C⁺, as compared to CD11B⁺CD11C⁻ resident alveolar macrophages (RAMs) (Qian et al., 2009). MAMs and their monocytic progenitors express CCR2 and respond to the CCL2 signal emitted from metastases (Lu & Kang, 2009; Qian et al., 2011). Once recruited to the metastatic site, MAMs can secrete vascular endothelial growth factor A to facilitate cancer cell extravasation for seeding of the lung (Qian et al., 2011). MAMs also secrete CCL3, which acts in an autocrine manner to augment MAM recruitment and enhance their activity to promote metastatic growth (Kitamura et al., 2015). Despite the significance of these findings, they represent only a patchwork of a global program of MAMs, which remains elusive. To this end, we performed deep RNA sequencing (RNA-seq) in MAMs isolated from micro-dissected lung metastases 11 days after tumor cell inoculation to gain unbiased insight into the global transcriptional program and to learn how MAMs may use this program to regulate metastatic growth in secondary organs.

Methods
Ethics statement and animal experiments
All procedures involving mice were conducted in accordance with National Institutes of Health regulations concerning the use and care of experimental animals and were approved by the Albert Einstein College of Medicine Animal Use Committee (animal protocol numbers: 20180202 and 20180205). Mice were housed and maintained in a barrier facility at the Albert Einstein College of Medicine and were kept on a 12-hour light/dark cycle and had ad libitum access to chow and water. The number of animals used in each experiment is explained in figure legends. A total of 66 mice were used. No animals were excluded from analyses. All efforts were made to ameliorate harm to animals. Mice carrying lung metastases were terminated by cervical dislocation after anesthesia with isoflurane before showing signs of difficulty breathing. Anesthesia with isoflurane was also performed for intravitral imaging as below.

C57BL6/J wild type (WT) mice were purchased from the Jackson Laboratory and were pooled together and were randomly assigned to the non-treated control group or the metastasis group. Lung metastases were induced by intravenous (iv) injection of 1 × 10⁶ E0771-LG cells (Kitamura et al., 2015) to the tail vein of syngeneic C57BL6/J female mice (6–8 weeks old) and were analyzed on day 11. Aif1⁻/⁻ mice were obtained by replacing all coding sequences with a modified LacZ gene in the 129SvEv background and were backcrossed into the C57BL6/J background for 14 generations by NS (Casimiro et al., 2013) and derived from his colony according to genotype.

For bone marrow transplantation (BMT), total bone marrow (BM) cells were extracted from 8–12 weeks old WT or Aif1⁻/⁻ female donor mice from their femurs, tibiae and spine by grinding in a mortar. Red blood cells were removed by incubation with RBC Lysis Buffer (Biolegend 420301) for 5 min at 4°C prior to injections. Female WT recipient mice of 4–6 weeks old were irradiated with 10 Gy gamma rays split into two doses with a four-hour interval (Mark I-68A 137Cs irradiator from JL Shepherd and Associates, San Fernando, CA). The random allocation of mice to experimental group (WT vs Aif1⁻/⁻) was driven by Mendelian Inheritance. The mice were then iv injected with 1 × 10⁶ BM cells from one of the donors the next day. Five weeks after BMT, lung metastases were induced by iv injection of 1 × 10⁶ E0771-LG-luciferase-ZsGreen cells (Zheng et al., 2019), and the mice were analyzed on day 10 by in vivo imaging system (IVIS) and histology.

For IVIS imaging, mice were anesthetized with isoflurane (2% v/v with oxygen as the carrier gas) in an inhalation chamber (VetEquipt 911103) before retro-orbital injection with d-luciferin in PBS (GoldBio, LUCK-1G, 1.5 mg/100 µL/20 g mouse). Afterwards, the mice were imaged using IVIS Spectrum In Vivo Imaging System (Perkin Elmer) while the anesthesia was maintained in the imaging chamber. Photon flux (photon/second/cm²/steradian) in the lung area was analyzed using Living Image Software (Perkin Elmer, v4.3.1; a possible free alternative is Aura Imaging Software from Spectral Instruments Imaging) and was expressed as relative values with respect to the lowest value in the WT group.

Flow cytometry and cell sorting
Lungs were perfused with PBS through the right ventricle, and metastases (<1 mm in diameter) were dissected under a dissection microscope and pooled from 1–5 mice. The tissues were subsequently digested using an enzyme mix of Liberase DL (Sigma-Aldrich 5466202001, 0.52 U/mL), TL (Sigma-Aldrich 5401020001, 0.26 U/mL) and DNase I (Sigma-Aldrich DN25, 150 µg/mL) for 30 min at 37°C. For sorting for RNA-seq or qRT-PCR, transcription inhibitors alpha-amanitin (Sigma-Aldrich A2263, 5 µg/mL) and actinomycin D (Sigma-Aldrich A1410, 1 µg/mL) were also added to the digestion buffer. Flow cytometry was analyzed using a LSRII cytometer (BD Biosciences), and the data were analyzed using Flowjo software (TreeStar, v10; a possible free alternative is Flowing Software 2.5.1 from University of Turku). FACSAria II (BD...
Biosciences) and Moflo Astrios (Beckman Coulter) were used for sorting. Antibody information is found in Table 1.

**RNA isolation, qRT-PCR and RNA sequencing**

Total RNAs were extracted using Picopure RNA Isolation kit (Arcturus KIT0202). RNA sequencing was performed at Beijing Genomic Institute, using the Ovation® RNA-Seq System V2 kit for library construction, pair-end 100 bp and Hiseq 4000, which generated 60–80M reads per sample. The reads were aligned to the mouse reference genome (GRCm38/mm10) using TopHat (v2.0.13) (Kim et al., 2013). Uniquely mapped reads were counted for each gene using htsq-cout in the HTSeq package (v0.6.1) with gene models from UCSC RefGene (Anders et al., 2015). FPKM values were generated using Cufflinks (v2.2.1) (Trapnell et al., 2013). Differentially expressed genes were identified using DESeq2 (Love et al., 2014). For qRT-PCR, RNAs were reverse transcribed and amplified using QuantiTect® Whole Transcriptome (Qiagen 207043) before qPCR. Gene expression was normalized to beta-actin. Relative expression is calculated using the formula –ddCt, where Ct stands for threshold cycles. See Table 2 for Taqman gene expression assays.

**Unsupervised hierarchical clustering, Gene Ontology, canonical pathway enrichment and gene set enrichment analysis**

For unsupervised hierarchical clustering, FPKM values were transformed by log2. Without prior grouping, genes of log2 (mean of FPKMs) > 5 and standard deviation > 1.5 were used for clustering. The differentially expressed genes (Extended Data: Table 3 (Zheng et al., 2021)) were analyzed for gene ontology annotation using the DAVID software (v6.8) (Huang et al., 2009) and the canonical pathway analysis using QIAGEN Ingenuity Pathway Analysis (QIAGEN IPA, March 2017 release; a possible free alternative is DAVID v6.8) (Krämer et al., 2014). For gene set enrichment analysis (GSEA), the MAM gene expression data were pre-ranked by the false-discovery rate (FDR) values: for upregulated genes, -log2 (FDR + A); for downregulated genes, log2 (FDR + A), and A = minimal FDR value that is not 0. This pre-ranked gene list was then analyzed in GSEA to interrogate if the three gene sets associated with IFN-alpha treatment of human macrophages (Greenwell-Wild et al., 2009; Liu et al., 2012; Tassiulas et al., 2004) as indicated in Figure 1 showed significant enrichment. GSEA (v3.0) was used for the analysis (Subramanian et al., 2005).

---

**Table 1. Antibodies used for flow cytometry, immunofluorescence and immunohistochemistry.**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Source</th>
<th>Identifier</th>
<th>Dilutions (/100 µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIF1</td>
<td>Abcam</td>
<td>Cat# ab178847</td>
<td>0.3</td>
</tr>
<tr>
<td>Anti-rabbit-ImmPRESS-HRP</td>
<td>Vector Laboratories</td>
<td>Cat# MP-6401-15</td>
<td>Ready-to-use</td>
</tr>
<tr>
<td>Anti-rat-ImmPRESS-HRP</td>
<td>Vector Laboratories</td>
<td>Cat# MP-7444, RRID:AB_2336530</td>
<td>Ready-to-use</td>
</tr>
<tr>
<td>CD11B, FITC, clone M1/70</td>
<td>BD Biosciences</td>
<td>Cat# 561688, RRID:AB_10898180</td>
<td>0.5</td>
</tr>
<tr>
<td>CD11B, PE-Cy7, clone M1/70</td>
<td>BioLegend</td>
<td>Cat# 101215, RRID:AB_312798</td>
<td>0.5</td>
</tr>
<tr>
<td>CD11B, PE, clone M1/70</td>
<td>BD Biosciences</td>
<td>Cat# 561689, RRID:AB_10893803</td>
<td>0.5</td>
</tr>
<tr>
<td>CD11C, BUv395, clone HL3</td>
<td>BD Biosciences</td>
<td>Cat# 564080, RRID:AB_2738580</td>
<td>1</td>
</tr>
<tr>
<td>CD11C, FITC, clone HL3</td>
<td>BD Biosciences</td>
<td>Cat# 561045, RRID:AB_10562385</td>
<td>1</td>
</tr>
<tr>
<td>CD45, APC-Fire750, clone 30-F11</td>
<td>BioLegend</td>
<td>Cat# 103153, RRID:AB_2572115</td>
<td>0.8</td>
</tr>
<tr>
<td>CD45, APC, clone 30-F11</td>
<td>BD Biosciences</td>
<td>Cat# 559864, RRID:AB_398672</td>
<td>0.8</td>
</tr>
<tr>
<td>CD45, PerCP-Cy5.5, clone 30-F11</td>
<td>BD Biosciences</td>
<td>Cat# 550994, RRID:AB_394003</td>
<td>0.8</td>
</tr>
<tr>
<td>Donkey anti-rabbit, AF594</td>
<td>ThermoFisher</td>
<td>Cat# A-21207, RRID:AB_141637</td>
<td>0.3</td>
</tr>
<tr>
<td>Donkey anti-rat-biotin</td>
<td>Jackson ImmunoResearch</td>
<td>Cat# 712-065-153</td>
<td>0.2</td>
</tr>
<tr>
<td>Donkey anti-rat, AF488</td>
<td>ThermoFisher</td>
<td>Cat# A-21208, RRID:AB_2535794</td>
<td>0.3</td>
</tr>
<tr>
<td>F4/80, AF647, clone 3A3-1</td>
<td>Bio-Rad</td>
<td>Cat# MCA497A647, RRID:AB_323931</td>
<td>6</td>
</tr>
<tr>
<td>LY6C, BV421, clone AL-21</td>
<td>BD Biosciences</td>
<td>Cat# 562727, RRID:AB_2737748</td>
<td>0.5</td>
</tr>
<tr>
<td>MRC1, PE-Cy7, clone C068C2</td>
<td>BioLegend</td>
<td>Cat# 141719, RRID:AB_2562247</td>
<td>1</td>
</tr>
<tr>
<td>MRC1, PE, clone C068C2</td>
<td>Biolegend</td>
<td>Cat# 141705, RRID:AB_10896421</td>
<td>1</td>
</tr>
<tr>
<td>Streptavidin, AF488</td>
<td>Jackson ImmunoResearch</td>
<td>Cat# 016-540-084</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Table 2. Information for the Taqman probes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Taqman assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>Mm02619580_g1</td>
</tr>
<tr>
<td>Aif1</td>
<td>Mm01132451_g1</td>
</tr>
<tr>
<td>Ifi35</td>
<td>Mm01260550_g1</td>
</tr>
<tr>
<td>Ifit1</td>
<td>Mm00515153_m1</td>
</tr>
<tr>
<td>Ifitm2</td>
<td>Mm00850080_g1</td>
</tr>
<tr>
<td>Ifitm3</td>
<td>Mm00847057_s1</td>
</tr>
<tr>
<td>Ifnar2</td>
<td>Mm00494916_m1</td>
</tr>
<tr>
<td>Irf1</td>
<td>Mm01288580_m1</td>
</tr>
<tr>
<td>Irf9</td>
<td>Mm00492679_m1</td>
</tr>
<tr>
<td>Isg15</td>
<td>Mm01705338_s1</td>
</tr>
<tr>
<td>Stat1</td>
<td>Mm01257286_m1</td>
</tr>
<tr>
<td>Stat2</td>
<td>Mm00490880_m1</td>
</tr>
<tr>
<td>Tap1</td>
<td>Mm00443188_m1</td>
</tr>
</tbody>
</table>

Immunofluorescence and immunohistochemistry

Briefly, lungs were perfused with 1% w/v PFA through the right ventricle, inflated with 2% w/v agarose in PBS via the trachea (for frozen sections) or 1% PFA (for paraffin sections), followed by immersion fixation in 4% w/v PFA for 1 hour at +4°C. For frozen embedding, the tissues were incubated in 25% w/v sucrose in PBS for 6 h or overnight before embedding in OCT Compound (Fisher HealthCare, 4585). Staining was performed in a similar manner to that previously published (Zheng et al., 2019). Briefly, 20-µm frozen sections or 5-µm paraffin sections of the lung were permeabilized with PBS containing 0.3% v/v Triton-X, blocked with PBS containing 0.3% v/v Triton-X, 5% v/v donkey serum, 0.05% w/v sodium azide and 0.2% w/v bovine serum albumin, and incubated with primary antibodies overnight. The sections were subsequently washed and incubated with secondary antibodies conjugated with fluorochromes or horseradish peroxidase (HRP). For HRP-conjugated secondary antibodies, the signal was visualized using ImmPACT® NovaRED® Substrate (Vector Laboratories, SK-4805). For F4/80 immunofluorescence, an additional amplification step using anti-rat-biotin and streptavidin-AF488 was performed. For antigen retrieval in immunohistochemistry of paraffin sections, Tris-EDTA (pH 9.0) (VWR, #K043) for AIF1 was used. See Table 1 for the antibody details.

Figure 1. Metastasis-associated macrophages (MAMs) recruited to lung metastases express a distinct gene expression program. (A) Gating strategy for isolating MAMs and resident alveolar macrophages (RAMs). Lung metastases (mets.) were induced by intravenous injection of E0771-LG cells, dissected and prepared for fluorescence-activated cell sorting (FACS) sorting for MAMs. RAMs from normal lungs were sorted similarly. (B) Quantification of the CD11B/CD11C population in panel A. Pool of three independent experiments including one for the qRT-PCR analysis in Figure 2B. N = 9 control mice and eight metastases samples pooled from 18 mice in total. ****, p <0.0001. (C) Sorted MAMs and RAMs were analyzed with RNA-sequencing, and the gene expression dataset was analyzed using unsupervised hierarchical clustering.
Quantification of lung metastases was performed similarly as previously described (Nielsen et al., 2001). Briefly, paraffin sections were stained with hematoxylin and scanned with the High Capacity Slide Scanner PANNORAMIC 250 Flash III (3D HistoCh). Images were analyzed with Fiji (NIH, v2.0.0-rc-64/1.51s), using the Colour Deconvolution and Analyze Particles functions, to quantify the total area of lung metastases and the total lung area. Eighteen mice (nine in each group) were analyzed for histology of lung metastases.

Statistical analysis
Each sample represents an individual mouse or pooled lung metastases obtained from 1–5 mice. Student’s t test or two-way ANOVA (for pooling of multiple experiments) were used. Statistical analyses were carried out with Graphpad Prism (version 7). For RNA-seq analysis, multiple hypothesis testing was adjusted using the Benjamini and Hochberg FDR method. Sample sizes were determined empirically.

Results
Gene expression profiling identifies strong activation of interferon signaling in MAMs
To analyze the gene expression profiles of MAMs and RAMs, we dissected metastases from affected lungs 11 days after seeding and normal lungs before cell dissociation for fluorescence-activated cell sorting (FACS) sorting of MAMs and RAMs. Consistent with previous findings, lung metastases were significantly enriched with the CD11B+CD11C+ subset of CD45+F4/80+LY6C-MRC1–FSC+ macrophages, as compared to the predominant CD11B+CD11C+ RAMs in normal lungs (Figure 1A and 1B). RNA-seq of these two populations revealed distinct gene expression programs between the two, as unsupervised hierarchical clustering completely segregated the two populations (Figure 1C).

Gene Ontology annotation of the genes differentially expressed by MAMs showed enrichment for cellular responses to cytokine stimulus and reduction in lipid metabolism (Extended Data: Tables 1–3 (Zheng et al., 2021)). To study the biological pathways enriched by the differentially expressed genes in more detail, we performed canonical pathway enrichment analysis using QIAGEN IPA (Extended Data: Table 4 (Zheng et al., 2021)). “Interferon (IFN) Signaling pathway” was the most enriched pathway and was predicted to be significantly activated (Figure 2A). An independent qRT-PCR analysis of the genes in this pathway confirmed the upregulation of most of the genes (Figure 2B and 2C). In addition, GSEA revealed that MAMs were strongly enriched for three independent gene sets obtained from IFN-alpha-treated human macrophages (Figure 2D). These data suggest that increased expression of the pathway components indeed translates into the signaling output of the pathway. Together with the additional significantly altered pathways such as the NF-kappa B pathway and various interleukin pathways (Extended Data: Table 5 (Zheng et al., 2021)), these findings suggest that MAMs recruited to lung metastases have a profoundly different gene expression program from RAMs indicative of unique functional roles.

AIF1 expression indicates MAM activation but is dispensable for regulation of lung metastases
To study if and how genes differentially expressed by MAMs may regulate lung metastasis, we focused on Aif1. It is one of the most upregulated genes (Log(fold change)>6, Extended Data: Table 3 (Zheng et al., 2021)) and is in two significantly enriched gene sets that are relevant to the current study (“response to interferon-gamma” and “leukocyte chemotaxis”) (Extended Data: Table 4 (Zheng et al., 2021)). GO:0034341 and GO:0030595). AIF1 is an intracellular calcium-binding and IFN-gamma-inducible protein mostly expressed in the monocyte/macrophage lineage (Zhao et al., 2013). It integrates various inflammatory stimuli, supports production of particular cytokines, and is critical for survival and pro-inflammatory activity of macrophages (Chinnasamy et al., 2015; Zhao et al., 2013). We verified with an independent qRT-PCR assay the dramatic upregulation of the Aif1 transcript in MAMs (Figure 3A). This finding was also corroborated by immunofluorescent staining for the AIF1 protein, which showed that approximately 40% of macrophages within metastatic nodules robustly expressed AIF1, whereas little was detected in RAMs in the normal lung (Figure 3B and 3C).

Next, we tested if induction of AIF1 in MAMs is required for promoting metastatic growth in the lung. To restrict Aif1 gene ablation to the hematopoietic lineage, we performed a bone marrow transplantation assay in which the bone marrow of Aif1 –/– donor mice (Casimiro et al., 2013) was transplanted into WT recipient mice. Lung metastases were induced in these mice for comparison with WT-to-WT recipient mice. Bioluminescent signals emitted from tumor cells that expressed luciferase revealed little difference between the two genotypes (Figure 3D and 3E). Histological evaluation of the metastases was consistent with the bioluminescent assay (Figure 3F and 3G). The lack of AIF1-expressing cells in metastases excluded the possibility of inefficient BM engraftment (Figure 3H). Together, these data suggest that AIF1 indicates MAM activation, but its expression by MAMs is dispensable for regulation of metastasis.

Discussion
In this study, we profiled the transcriptome of MAMs by deep RNA-seq and identified a distinct expression program that are indicative of important metastasis-regulatory functions.

IFN signaling has been shown to have anti-metastatic effects via inhibition of epithelial-to-mesenchymal transition of tumor cells, angiogenesis, intravasation, survival in the circulation, homing to target tissues, and extravasation (Ortiz & Fuchs, 2017). The effects of IFN treatment on MAMs with respect to regulation of metastasis are, however, unknown. Given that ablation of MAMs or inhibition of MAM recruitment suppressed metastatic growth in multiple models (Lu & Kang, 2009; Nielsen et al., 2016; Qian et al., 2011; Qian et al., 2009), it is plausible that activation of IFN signaling promotes the pro-metastatic activity of MAMs, which may therefore limit the favorable effects of IFN-alpha treatment for patients of metastatic.
Figure 2. Activation of the interferon pathway in metastasis-associated macrophages (MAMs). (A) Top 10 canonical pathways enriched by the differentially expressed genes in MAMs as analyzed using Ingenuity Pathway Analysis (IPA). (B) qRT-PCR analysis of resident alveolar macrophages (RAMs) and MAMs for genes in the Interferon Signaling pathway. Mean +/- standard error of the mean N = 3 control mice and three lung metastasis samples pooled from eight mice. ****, p < 0.0001; ***, p < 0.001; **, p < 0.01; *, p <0.05. (C) Schematic representation of the activation of the Interferon Signaling pathway. Shades of redness indicate the extent of upregulation as detected by the RNA-sequencing. #, significantly upregulated genes as validated in panel B are marked here. (D) Gene set enrichment analysis showed enrichments of three gene sets from IFN-alpha-treated human macrophages in MAMs, indicating a strong interferon signature in MAMs compared to RAMs.

diseases (Eggermont et al., 2014). In support of this hypothesis, STAT1, an essential signaling molecule of the IFN pathway, in tumor-associated macrophages regulates the T cell-suppressive activity of tumor-associated macrophages (Kusmartsev & Gabrilovich, 2005). It has been suggested that the difficulty to target the right IFNs at the right dose in the right type of cells might underlie the basis for the limited therapeutic effects of IFNs (Dunn et al., 2006). It is thus interesting to determine in future work if macrophage-specific inactivation of IFN signaling inhibits development of metastasis, and more importantly if MAM-targeting will improve IFN therapy and immunotherapy.

Despite robust induction of the interferon-inducible gene Aif1 in MAMs, we did not see an effect of AIF1 deficiency on
AIF expression is induced in metastasis-associated macrophages (MAMs) but is dispensable for regulation of metastatic growth in the lung. (A) qRT-PCR analysis of \textit{Aif1} in resident alveolar macrophages (RAMs) and MAMs. The same mice and samples were used as in Figure 2B. **, \( p < 0.01 \). (B) Immunofluorescent staining of lung sections from normal mice and mice bearing lung metastases induced by E0771-LG intravenous (iv) injection. Arrowheads, F4/80\(^+\) macrophages negative for AIF1. Arrows, F4/80\(^+\) macrophages positive for AIF1. White boxes in column 4 are magnified in columns 1–3. Scale bars = 20 µm in columns 1-3 and 50 µm in column 4. (C) Quantification of F4/80-AIF1\(^+\) cells in total F4/80\(^+\) cells in panel B. N = 4 control mice and 4 metastasis-bearing mice. ***, \( p < 0.001 \). (D) Lethally irradiated wild-type (WT) recipient mice that received bone marrow transplantation from WT or Aif1\(^{-/-}\) mice were injected iv with E0771-LG and were analyzed for lung metastases using \textit{in vivo} imaging system imaging after 10 days. (E) Quantification of the bioluminescent signal from panel D. Pool of two experiments. Total N = 16 WT mice and 15 Aif1\(^{-/-}\) mice pooled from two independent experiments. N.S., not significant. (F) Hematoxylin staining of lung sections from mice in panel D. Scale bars = 500 µm. (G) Quantification of the total area of metastatic nodules per mouse normalized to total lung area in panel F. N = 9 mice in each group pooled from two independent experiments. N.S., not significant. (H) Immunohistochemistry for AIF1 of the lung sections from panel F. Dark brown indicates positive signal. Scale bars = 50 µm.

development of lung metastases. Similar strong correlation of AIF1-expressing macrophages/microglial cells with malignancy has been previously observed for human gliomas (Deininger et al., 2000). Another study shows that AIF1 is expressed by brain-infiltrating myeloid cells, which enhance metastatic growth by promoting proliferation and reducing apoptosis of tumor cells (Zhang et al., 2015). Functional testing in neither study has been attempted. It is thus interesting to determine whether AIF1 expression in macrophages or microglia plays a functional role in these settings or serves as a marker that indicates cell reprogramming in the tumor microenvironment as identified in our study.

In conclusion, we have identified a distinct gene expression program in MAMs that include strong activation of the IFN pathway and AIF1. Since cancer cells at metastatic sites benefit from these recruited MAMs, the gene expression profile revealed in this study can provide an important platform to uncover novel mechanisms and potential therapeutic targets that are essential for MAMs to promote metastatic growth.
**Data availability**

**Underlying data**


This project contains the following underlying data:

- Fig. 1A and B: FCS files for the FACS data and the values for the graph in panel B in XLSX format.
- Fig. 2B: ddCt values for the qPCR analysis of the IFN pathway.
- Fig. 3A: Ct values for the Aif1 qPCR analysis.
- Fig. 3B and C: Raw images of the fluorescence and values for the graph.
- Fig. 3D and E: Raw IVIS images and values for the graph.
- Fig. 3F: Raw images for histological evaluation of lung metastases and values for the graph.
- Fig. 3H: Raw images for AIF1 immunohistochemistry.

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

**Extended data**


This project contains the following extended data:

1. **Extended Data Table 1**: Fragments per kilobase of transcript per million mapped reads (FPKM) values of MAMs and RAMs. Uniquely mapped reads were counted for each gene using htsq-count in the HTSeq package (v0.6.1) with gene models from UCSC RefGene.

2. **Extended Data Table 2**: Differentially expressed genes as identified using DESeq2. Mean FPKM values < 1 in both groups under comparison were excluded from differential expression analysis. Fold change >2, FDR < 0.05.

3. **Extended Data Table 3**: Highly differentially expressed genes for IPA analysis. This gene set is obtained with the intersection of Extended Data Table 1 and 2, which is subsequently applied with the following more stringent criteria: log2 (fold change) > 1.5, FDR < 0.03; for upregulated genes, mean FPKMs of MAMs > 20; for downregulated genes, mean FPKMs of RAMs > 40.

4. **Extended Data Table 4**: Gene Ontology annotation of the highly differentially expressed genes. Upregulated and downregulated genes from Extended Data Table 3 were analyzed by the GOBP5 analysis of DAVID Bioinformatics Resources.

5. **Extended Data Table 5**: Canonical Pathways in IPA enriched by the differentially expressed genes in MAMs. Output for the Canonical Pathways in IPA using the highly differentially expressed genes from Extended Data Table 3.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

**Acknowledgements**

We would like to acknowledge support from the Analytical Imaging Facility, which is funded by the NCI Cancer Grant P30CA013330. The Leica SP8 confocal microscope was funded by the NIH 1S10OD023591-01, 3D Histech P250 High Capacity Slide Scanner by NIH 1S10OD019961-01, and IVIS Spectrum Imaging System by NIH 1S10RR027308-01.

---

**References**


Huang DW, Sherman BT, Lempicki RA: Systematic and integrative analysis of Wellcome Open Research 2021, 6:52 Last updated: 09 MAR 2021