

FABP4-mediated lipid accumulation and lipolysis in tumor associated macrophages promote breast cancer metastasis

Reviewed Preprint

v1 • October 1, 2024

Not revised

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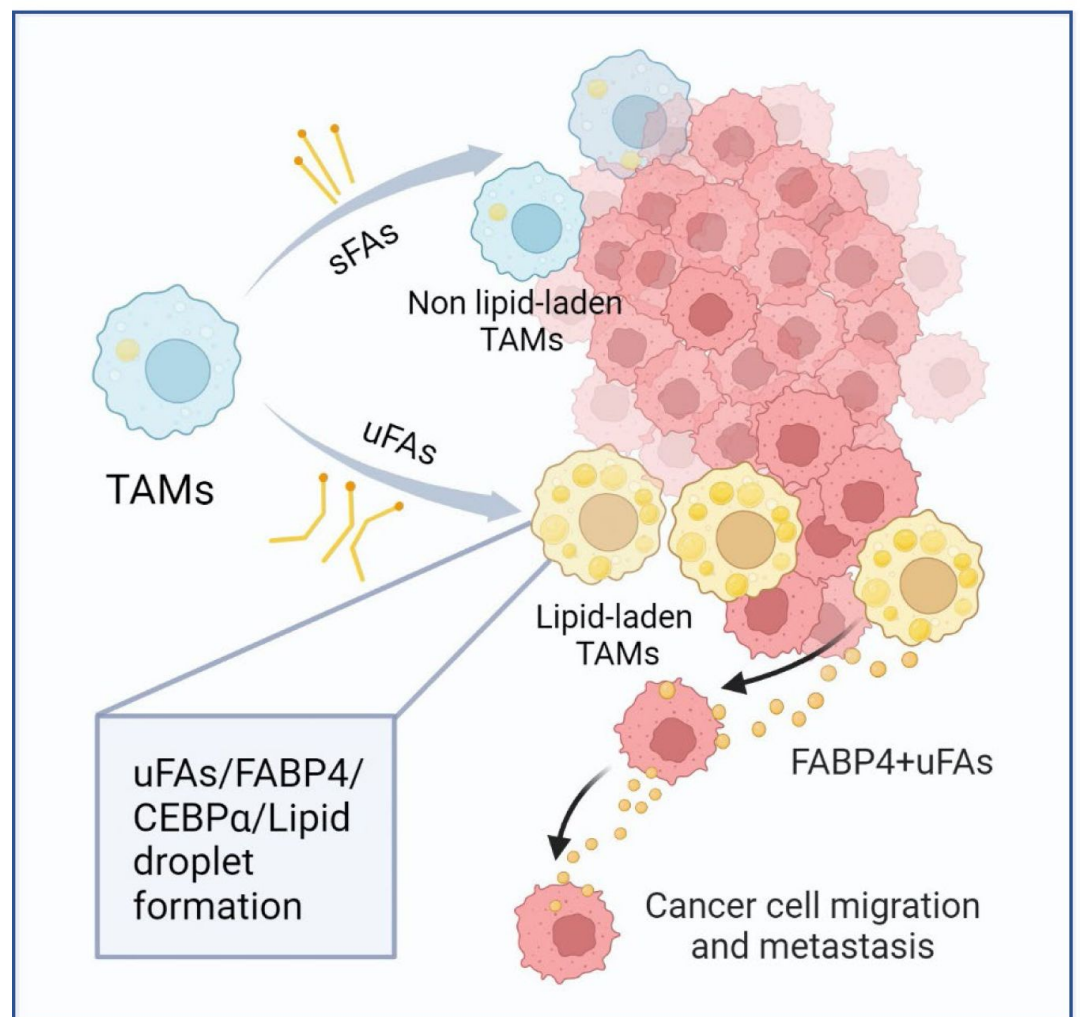
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Abstract

A high density of tumor-associated macrophages (TAMs) is associated with poorer prognosis and survival in breast cancer patients. Recent studies have shown that lipid accumulation in TAMs can promote tumor growth and metastasis in various models. However, the specific molecular mechanisms that drive lipid accumulation and tumor progression in TAMs remain largely unknown. Herein, we demonstrated that unsaturated fatty acids (FAs), unlike saturated ones, are more likely to form lipid droplets in macrophages. Specifically, unsaturated FAs, including linoleic acids (LA), activate the FABP4/CEBP α pathway, leading to triglyceride synthesis and lipid droplet formation. Furthermore, FABP4 enhances lipolysis and FA utilization by breast cancer cells, which promotes cancer cell migration *in vitro* and metastasis *in vivo*. Notably, a deficiency of FABP4 in macrophages significantly reduces LA-induced lipid metabolism. Therefore, our findings suggest FABP4 as a crucial lipid messenger that facilitates unsaturated FA-mediated lipid accumulation and lipolysis in TAMs, thus contributing to the metastasis of breast cancer.

Graphic Abstract



Highlights

1. Unlike saturated fatty acids, unsaturated fatty acids preferentially promote lipid droplet formation in macrophages.
2. Unsaturated fatty acids activate the FABP4/CEBPα axis for neutral lipid biosynthesis in macrophages
3. Deficiency of FABP4 compromised unsaturated fatty acid-mediated lipid accumulation and utilization in macrophages
4. FABP4-mediated lipid metabolism in macrophages contributes to breast cancer metastasis

eLife Assessment

This **important** paper uses elegant models, including genetic knock outs, to demonstrate that FABP4 contributes to lipid accumulation in tumor-associated macrophages, which seems to increase breast cancer migration. While the work is of high interest, the strength of the evidence relating to some of the conclusions is **incomplete** and the paper would benefit from some refinement. The work will be of interest to those researchers trying to link metabolism, the immune system, and cancer.

<https://doi.org/10.7554/eLife.101221.1.sa4>

Introduction

As a main arm of innate immunity, macrophages are present in almost all tissues of an organism, where they play pivotal roles in maintaining tissue homeostasis or contributing to disease pathogenesis¹. In breast cancer, tumor associated macrophages (TAMs) are the most abundant immune cells in the tumor microenvironment (TME), actively participating in tumor progression and metastasis^{2–4}. A high presence of TAMs in the TME correlates with a poorer prognosis of cancer patients in clinical studies^{5–7}. Moreover, macrophage deletion via genetic or therapeutic approaches results in inhibition and regression of mammary tumors in pre-clinical studies^{8–10}. These ample lines of evidence corroborate a crucial role for macrophages in facilitating breast cancer progression.

Macrophages are known for their heterogeneity regarding origin, phenotypes, and functional states¹¹. In response to diverse environmental stimuli from the environment, macrophages are believed to acquire a spectrum of functional states *in vivo*^{12,13}. To understand their functional versatility, macrophages are commonly divided into the M1/M2 paradigm based on the concept of adaptive Th1/Th2 polarization^{14,15}. Generally, Th1-derived cytokines polarize macrophages to M1 phenotype, which express inducible nitric oxide synthase and exhibit anti-tumor activities. Th2-cytokines polarized M2 macrophages highly express arginase 1, CD206, VEGFs, IL-10, TGFβ, *etc.*, supporting angiogenesis and favoring tumor growth^{16,17}. However, given the complex functional states of macrophages *in vivo*¹⁸, the M1/M2 classification is likely oversimplified. Many studies demonstrate that TAMs do not follow the two extreme M1/M2 activation states *in vivo*^{19,20}. Considering that various extrinsic and intrinsic factors in the TME can cooperatively shape TAM phenotypes and functions^{21,22}, identification of these factors is essential for targeting TAM-mediated cancer immunotherapy.

There is a growing realization that macrophage metabolism determines their functional outcome^{23,24}. Several recent studies in multiple cancer models, including breast cancer^{25–28}, reported that lipid accumulation in TAMs promotes tumor growth and metastasis. Specifically, lipid accumulation promoted the expression of Arg1, VEGFα, MMP9, IL-6, PD-L1, CCL-6, *etc.*, thus conferring TAMs with immune suppressive and pro-tumorigenic phenotypes. Lipid droplet formation was as a characteristic feature of the pro-tumor TAMs, but the underlying molecular mechanisms driving lipid droplet formation in TAMs remain to be determined.

Despite morphological and compositional differences, lipid droplets are all formed by a phospholipid monolayer enclosing a neutral lipid core²⁹. A key step of lipid droplet formation is to synthesize neutral triacylglycerol (TAG), which requires the esterification of activated fatty acids (FA) to diacylglycerol (DAG)³⁰. During this step, enzymes including glycerol-3-phosphate

acyltransferase (GPAT), acyl-CoA:1-acyl-glycerol-3-phosphate acyltransferase (AGPAT), DAG acyltransferases (DGATs), are key in the biogenesis of neutral lipids. As such, factors engaging with FA transport and regulating key enzyme activity are deemed to be critical for lipid droplet formation in TAMs.

Due to the insolubility of hydrophobic FAs, fatty acid binding proteins (FABPs) have evolved to solubilize FAs, facilitating their transport and responses³¹. Among FABPs, adipose FABP (A-FABP or FABP4) is the most well studied given its striking biology in regulating macrophage lipid metabolism and functions in multiple disease settings^{1,32}. Our pre-clinical studies demonstrated that a subset of macrophages with the phenotype of CD11b⁺F4/80⁺Ly6C[−]CD11c[−]MHCII[−] highly express FABP4, facilitating macrophage lipid processing and patrolling functions^{1,33}. Moreover, FABP4 was identified as a functional marker for protumor TAMs by enhancing the oncogenic IL-6/STAT3 signaling¹⁹. Given the emerging role of lipid accumulation in protumor TAMs, we hypothesized that FABP4 might function as a key molecular sensor promoting lipid accumulation and utilization in TAMs.

Utilizing a combination of *in vitro* cellular studies with various species of FAs, *in vivo* macrophage single-cell sequencing in mouse models, and macrophage/FABP4 analysis in human breast cancer specimens, we demonstrate that FABP4 plays a pivotal role in mediating lipid droplet formation and subsequent lipolysis for tumor utilization, thus contributing to TAM-mediated tumor growth and metastasis in breast cancer.

Results

Unsaturated FAs promote lipid accumulation in macrophages

Hostile TME, especially in breast cancer, is characterized by enriched FAs and other lipid species, which foster immunosuppression and support tumor growth and metastasis^{34,35}. Since palmitic acid (PA, 16:0), stearic acid (SA, 18:0), oleic acid (OA, 18:1), and linoleic acid (LA, 18:2) are the most common FAs in humans, as shown in **Figure 1A**, we treated macrophages with individual albumin-conjugated FAs and determined their fate. Using a macrophage cell line, we demonstrated that all the FAs studied were oxidized by mitochondria as evidenced by the increased oxygen consumption rate (OCR) when compared to the bovine serum albumin (BSA) control (**Figure 1B**). Interestingly, when measuring their impact on other cellular organelles, FAs exhibited dichotomous effects, with saturated FAs increasing lysosome contents (**Figure 1C**), while unsaturated FAs promoting ER (**Figure 1D**) and, more dramatically, lipid droplet (LD) formation (**Figure 1E**) in macrophages. Using primary peritoneal macrophages (**Figure S1A**) and bone marrow-derived macrophages (**Figure S1B**), we observed the similar phenomenon that unlike saturated FA, unsaturated FA significantly induced LD formation in these macrophages. Oil Red O staining confirmed the presence of LD formation (**Figure S1C**). Of note, the observation of unsaturated FA-induced LD formation in macrophages was consistent regardless of FA concentrations (**Figure S1D**) and treatment durations (**Figure S1E**). With a multispectral imaging analysis, we demonstrated that OA/LA-induced LD accumulation was not associated with the lysosome, but colocalized in the ER (**Figure 1F**). Transmission electron microscope further confirmed that OA and LA specifically induced LD formation in the cytoplasm (**Figure 1G**), which were bud from ER (**Figure S1F**). Collectively, these data suggest that unsaturated FA-induced LD biogenesis in macrophages mainly occurs in the ER.

Unsaturated FAs upregulate key enzymes for LD biogenesis

It is well known that key enzymes, including GPATs, AGPATs, LIPINs, DGATs, are involved in TAG synthesis and LD biogenesis (**Figure 2A**). To determine how unsaturated FAs, but not saturated FAs, induced LD biogenesis in macrophages, we treated macrophages with either LA or PA, and

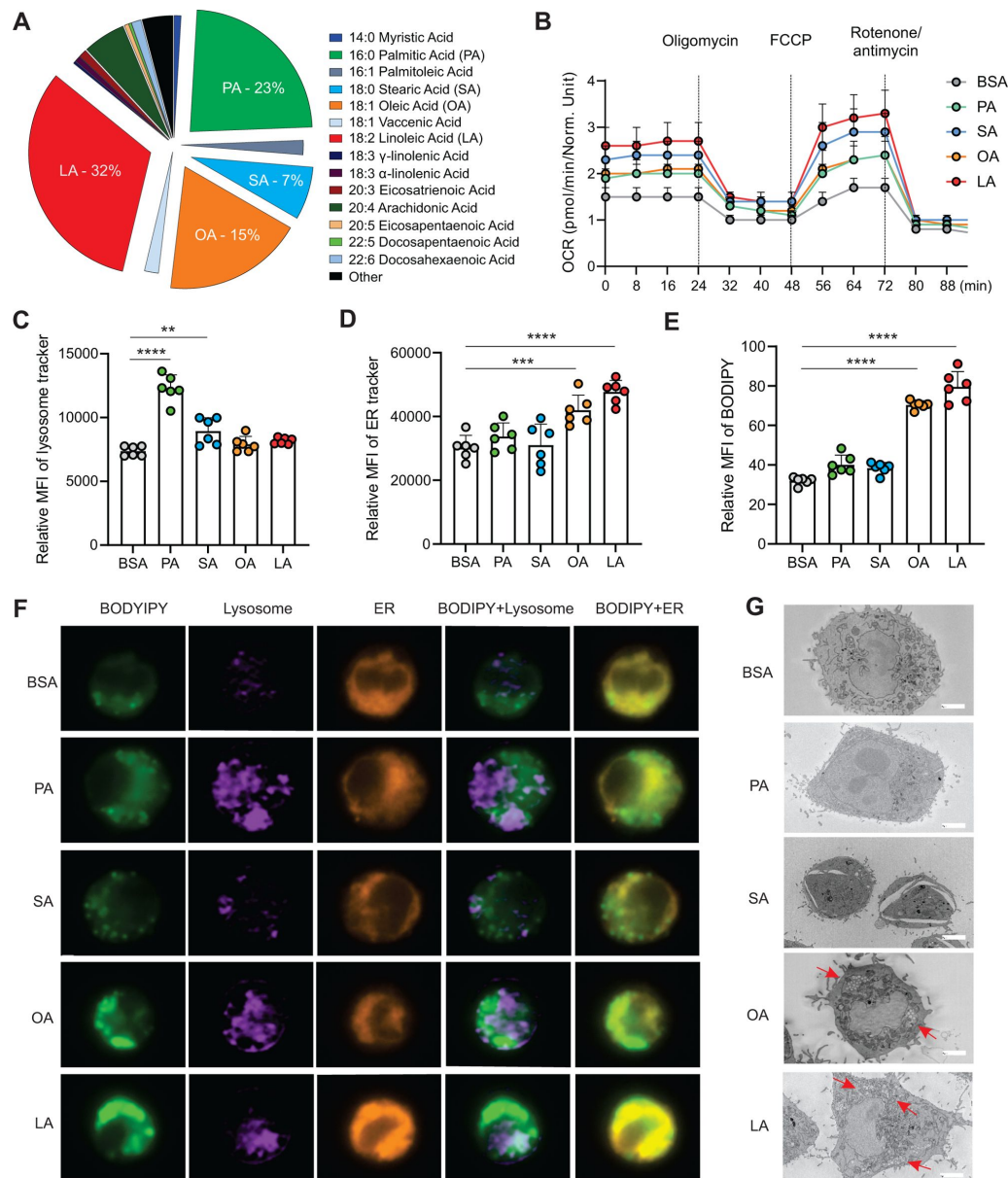


Figure 1

Unsaturation FAs form lipid accumulation in macrophages

A, Pie chart showing main compositions of free fatty acids in the serum of healthy humans

B, Measurement of oxygen consumption rate (OCR) in macrophages treated with 200 μ M of PA, SA, OA, LA or control BSA, respectively, under basal conditions or following the addition of oligomycin, FCCP or the electron transport inhibitor Rotenone/antimycin by a seahorse XF-96 analyzer (n=5).

C-E, Macrophages were treated with 200 μ M of PA, SA, OA, LA or BSA for 4h. Flow cytometric analysis of lysosome, ER and lipid droplet formation by measuring mean fluorescent intensity (MFI) of lysosome tracker (C), ER tracker (D) and BODIPY (E) in macrophages.

F, Multispectral imaging analysis of BODIPY (green), Lysosome (purple), ER (orange), and merged images showing the colocalization status of BODIPY/lyso-tracker and BODIPY/ER-tracker in macrophages treated with PA, SA, OA, LA or BSA at 200 μ M for 4h.

G, Analysis of lipid droplet formation (red arrow) in macrophages treated with BSA, PA, SA, OA and LA by transmission electron microscope.

Data are shown as mean $\pm$ SD in panel B-E (** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, as compared to the control BSA group, unpaired Student t test). See also Figure S1.

measured their impact on LD biogenesis-related enzymes. In response to PA or LA treatment, Acs1, Cpt1a, Cpt1b, HMGCR, Acat1 and Acat2 had similar expression pattern in macrophages (Figure S2A-S2F), suggesting that both FA types exerted similar effects on FA activation, mitochondrial oxidation, cholesterol synthesis and esterification. Interestingly, genes encoding rate-limiting enzymes in TAG synthesis, including Gpm1, Dgat1 and Dgat2, were significantly increased by treatment with LA, but not PA (Figure 2B–2H), suggesting a unique role of LA in transcriptional upregulation of neutral lipid synthesis pathways. We observed by confocal microscopy that macrophages treated with LA, but not BSA or PA, exhibited elevated levels of LipidTOX, GPAT1 and DGAT1 (Figure 2I). Quantitative measurements showed a significant increase of lipid accumulation (LipidTOX), GPAT1 and DGAT1 proteins in the LA-treated vs. the BSA or PA groups (Figure 2J–2M). Moreover, intracellular flow cytometric staining (Figure S2G-S2I) and quantification by mean fluorescent intensity (MFI) (Figure S2J-S2L) also confirmed our observations that unsaturated FAs significantly increased lipid accumulation by enhancing GPAT/DGAT-mediated lipid accumulation in macrophages.

C/EBP α transcriptionally controls LA-induced TAG synthesis

To determine how unsaturated LA transcriptionally upregulated gene expression in the TAG synthesis pathway, we searched for transcription factor binding sites, focusing on the pooled genes related to TAG synthesis pathways. Two transcriptional factors, C/EBP α and C/EBP β , were predicted to commonly bind the promotor regions of these genes (Figure S3A). When macrophages were treated with PA or LA, C/EBP α (Figure 3A), but not C/EBP β (Figure S3B), was significantly upregulated by LA treatment. Confocal microscopy showed that C/EBP α was mainly localized in the cytosol with BSA and PA treatment but was significantly upregulated in the nuclei with LA treatment (Figure 3B, 3C). In contrast, C/EBP β was present in both cytosol and nuclei regardless of PA or LA treatment (Figure S3C, S3D). These results suggest that LA treatment induces specific activation of C/EBP α in macrophages.

To further dissect the role of C/EBPs in LA-treated macrophages, we knocked down C/EBP α (Figure 3D) and C/EBP β (Figure S3E), respectively, in macrophages. LA-induced gene expression of Gpm1, Dgat1, Dgat2 was abrogated when C/EBP α was silenced (Figure 3E–3G). Moreover, the uptake of FA (Cd36), lysosome lipase (Lipa) and mitochondrial oxidation (Cpt1b) were significantly increased in C/EBP α -knockdown macrophages (Figure 3H–3J), suggesting that C/EBP α knockdown not only inhibited lipid synthesis, but also promoted lipolysis and oxidation. As such, C/EBP α knockdown significantly inhibited LA-induced neutral lipid accumulation as shown by the staining of BODIPY (Figure 3K) and Oil Red O (Figure 3L, 3M) in macrophages. By contrast, C/EBP β silencing did not affect the expression of Gpm1, Cd36, and Lipa expression (Figure S3F-S3H). Although C/EBP β knockdown appeared to inhibit the expression of Dgat1 and Dgat2, it also inhibited Cpt1b-mediated FA oxidation (Figure S3I-S3K). Overall, C/EBP β knockdown resulted in lipid accumulation in response to LA treatment in macrophages (Figure S3L). Altogether, these data suggest that C/EBP α , but not C/EBP β , is a critical transcriptional factor controlling LA-induced TAG synthesis and LD formation in macrophages.

FABP4 mediates LA-induced C/EBP α activation

Given the insolubility of long chain FAs, it was of great interest to dissect how LA, unlike SA, specifically activated C/EBP α in macrophages. As FA chaperones, FABPs bind different FAs, facilitating their transport and responses. We measured the profile of common FABP members in macrophages and demonstrated that FA treatment mainly induced expression of FABP4, but not FABP3 and FABP5, in macrophages (Figure 4A–4C). Analyzing the TCGA human breast cancer database, we found that compared to other FABP family members FABP4 was mostly correlated with C/EBP α (Figure S4A, S4B), but not with other C/EBP members (Figure S4C). The positive associations of FABP4 with C/EBP α and key TAG synthesis genes were also evidenced in multiple GEO datasets (Figure S4D-S4F).

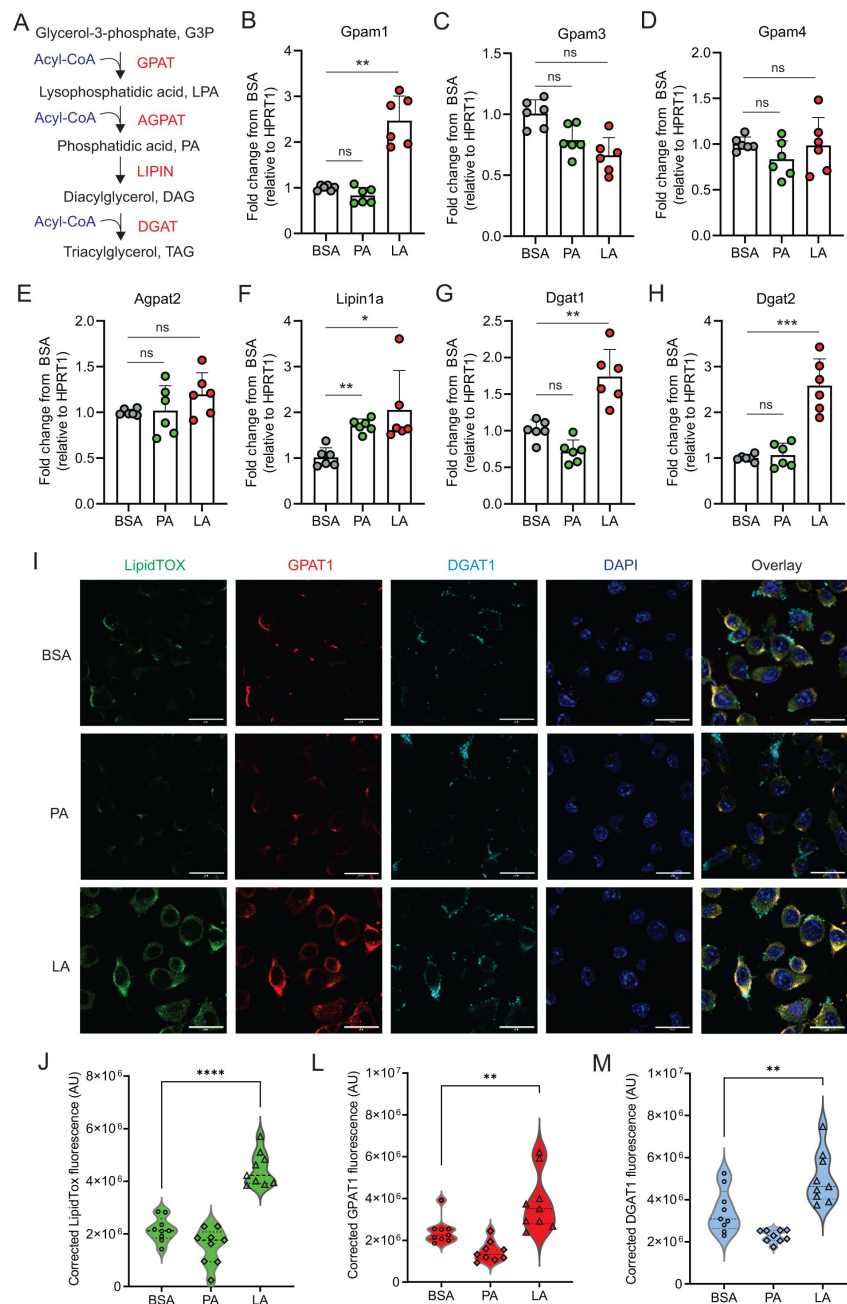


Figure 2

LA induces the expression of key enzymes of triacylglycerol synthesis in macrophages

A, Key enzymes in the biosynthesis of triacylglycerol.

B-H, Analyzing the expression of genes encoding key enzymes, including Gpm1 (B), Gpm3 (C), Gpm4 (D), Agpat2 (E), Lipin1a (F), Dgat1 (G), Dgat2 (H), in the triacylglycerol biosynthesis pathway in macrophages treated with 400μM of PA, LA or BSA for 4 hours by real-time PCR.

I, Representative confocal images of lipid accumulation by LipidTOX staining (green), expression of GPAT1 (red), DGAT1 (blue) in macrophages treated with BSA, PA and LA (400uM) overnight.

J-M, Quantification of lipid accumulation (J), protein levels of GPAT1 (K) and DGAT1 (M) in macrophages treated with BSA, PA or LA (400μM) overnight.

Data are shown as mean ± SD in panel B-H, J-M (** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, ns, non-significant, as compared to the control BSA group, unpaired Student t test). See also Figure S2.

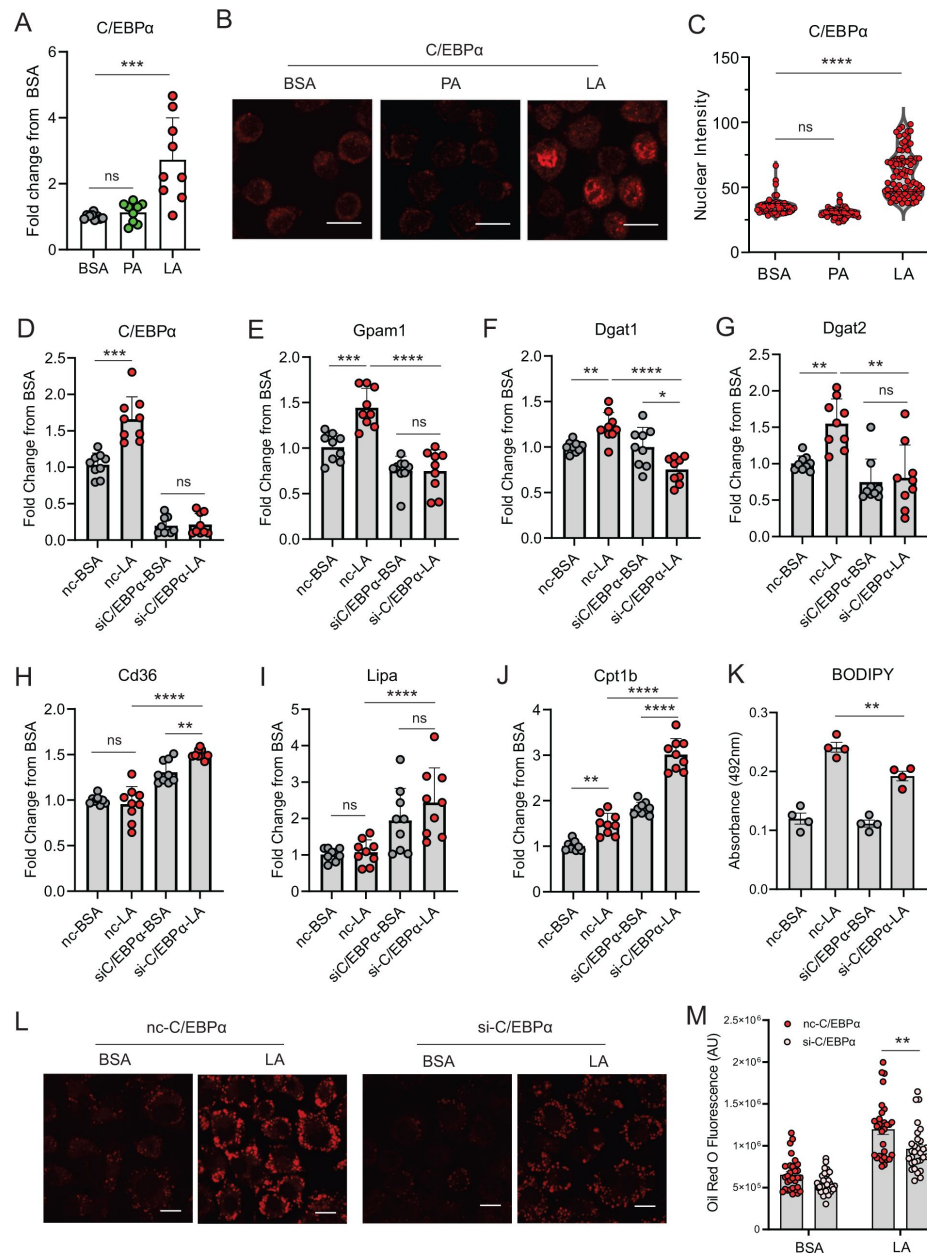


Figure 3

LA induces lipid accumulation through activating the C/EBPα pathway

A, Measurement of C/EBPα gene expression levels in macrophages treated with BSA, PA, or LA (400μM) for 4 hours by real-time PCR.

B, Representative confocal images of C/EBPα protein expression (red) in macrophages treated with BSA, PA, or LA overnight.

C, Quantification of C/EBPα nuclear expression in macrophages treated with BSA, PA, or LA overnight by Image J analysis.

D-J, Real-time PCR analysis of the levels of C/EBPα (D), Gpam1 (E), Dgat1 (F), Dgat2 (G), Cd36 (H), Lipa (I), and Cpt1b (J) in macrophages transfected with 40 nM C/EBPα siRNA or control siNC and then treated with BSA or LA for 4 hours.

K, Measurement of BODIPY fluorescent intensity in C/EBPα-silencing or control macrophages treated with BSA or LA using a fluorescence spectroscopy.

L, Representative confocal images of Oil Red O staining in C/EBPα-silencing or control macrophages treated with BSA or LA (bar, 10μM).

M, Quantification of Oil Red O fluorescence intensity in C/EBPα-silencing or control macrophages treated with BSA or LA.

Data are shown as mean ± SD in panel A, C-K and M (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001, ns, non-significant, as compared to the control BSA group or control siNC group, unpaired Student t test). See also Figure S3.

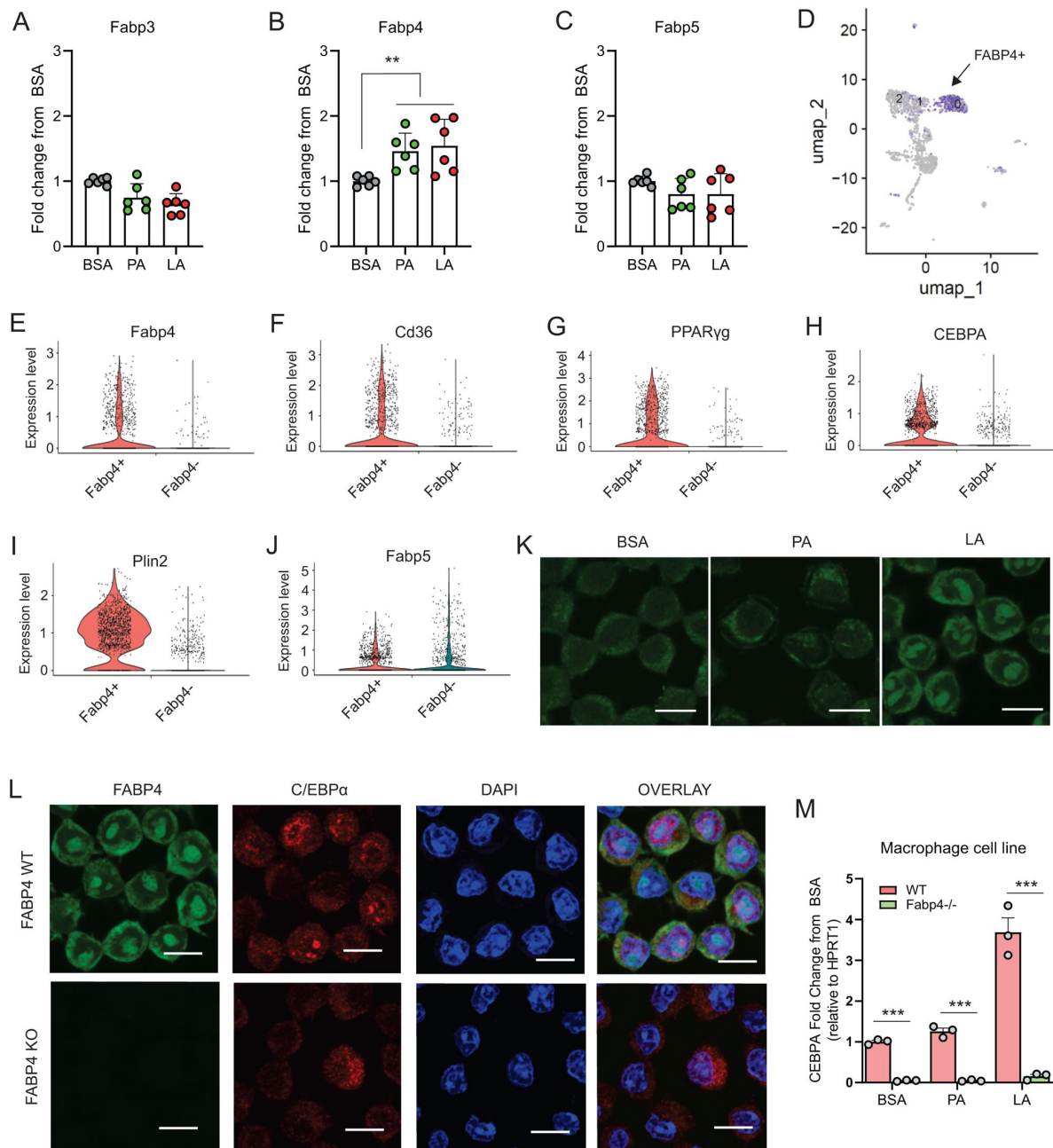


Figure 4

FABP4 mediates LA-induced C/EBPα expression in macrophages

A-C, Analysis of the expression of FABP family members, including Fabp3 (A), Fabp4 (B) and Fabp5 (C), in macrophages treated with BSA, PA or LA (400μM) for 4 hours.

D, UMAP of FABP4-positive macrophage subsets using mouse spleen single-cell RNA sequence analysis.

E-J, Violin plots showing relative expression levels of genes, including Fabp4 (E), Cd36 (F), PPARγ (G), CEBPA (H), Plin2 (I) and Fabp5 (J) between Fabp4+ vs Fabp4- macrophages indicated in (C).

K, Confocal analysis of FABP4 expression in macrophages treated with BSA, PA or LA (400uM) (bar, 10μM).

L, Comparison of the expression of FABP4 and C/EBPα between FABP4 wildtype (WT) and knockout (KO) macrophages in response to LA treatment (400μM) (bar, 10μM).

M, Realtime PCR analysis of CEBPA expression in WT and FABP4^{-/-} macrophages treated with BSA, PA, and LA (400μM).

Data are shown as mean ± SD in panel A and L (** p ≤ 0.01, *** p ≤ 0.001, as compared to the control BSA group or FABP4^{-/-} group, unpaired Student t test). See also Figure S4.

To gain insights into our observations, we performed single cell RNA-sequencing using splenic macrophages. UMAP plots showed that macrophages consisted of 13 clusters based on untargeted gene expression profiles (Figure S4G). FABP4 was enriched in clusters 0, 1 and 2 (**Figure 4D** [↗](#)). Compared to other non-enriched clusters (Fabp4⁻), GO molecular function analysis showed that FABP4 was mainly involved in protein-lipid complex, lipid binding and signaling receptors (Figure S4H). Moreover, Fabp4 enriched clusters (Fabp4⁺) were highly associated with genes known to be critical for lipid uptake and accumulation, including Cd36, PPAR γ , CEBPA, Plin2 (**Figure 4E** [↗](#)-**4I** [↗](#)). In contrast, Fabp4 enrichment was not related to Fabp5, Acsl1, Lipa, Fasn and Cpt1b (**Figure 4J** [↗](#), Figure S4I-S4L), suggesting a unique role of FABP4 in lipid uptake and storage in macrophages.

Confocal microscopy showed that FABP4 was mainly expressed in the cytosol in BSA- or PA-treated cells, whereas LA treatment led to FABP4 present both in the cytosol and nuclei (**Figure 4K** [↗](#)), suggesting a LA-induced FABP4 nuclear translocation effect for potential nuclear transactivation. To confirm the role of FABP4, we measured C/EBP α expression in FABP4-deficient macrophages. Deletion of FABP4 in macrophages abrogated LA-induced C/EBP α expression in nuclei (**Figure 4L** [↗](#), **4M** [↗](#)). We further confirmed that FABP4 deficiency significantly blocked LA-induced expression of C/EBP α (Figure S4M, S4N), but not C/EBP β (Figure S4O, S4P), in primary peritoneal macrophages and bone marrow-derived macrophages. Collectively, these data demonstrated a pivotal role for FABP4 in mediating LA-induced C/EBP α activation in macrophages.

FABP4 expression enhances lipid accumulation in macrophages

Given the critical role of FABP4 in activating the LA-C/EBP α axis, we reasoned that FABP4 deficiency suppressed LA-induced lipid accumulation in macrophages. To this end, we first measured if FABP4 deficiency reduced key enzymes of TAG synthesis in different macrophages. Using FABP4 wild type (WT) and knockout (KO) macrophage cell lines (**Figure 5A** [↗](#)), we demonstrated that upregulation of Gpm1, Dgat1, and Dgat2, but not Gpm4 and Agpat2, in response to LA treatment in WT macrophages, was significantly reduced when FABP4 was genetically depleted (**Figure 5B** [↗](#)-**5F** [↗](#)). Similarly, using peritoneal macrophages purified from WT and KO mice, we confirmed that FABP4 deficiency significantly reduced the expression of LA-induced Gpm1, Dgat1, Dgat2 (Figure S5A-S5D). Of note, FABP4 deficiency also reduced expression of other lipid transporters, including Cd36 and Fabp5 (Figure S5E-S5F), suggesting a general inhibition of fatty acid uptake and metabolism in the absence of FABP4 in primary macrophages.

To evaluate the role of FABP4 in lipid accumulation, we measured GPAT1 and DGAT1 protein expression and lipid droplet formation in LA-treated WT and KO macrophages. Confocal microscopy showed that GPAT1, DGAT1 and lipidTOX were colocalized in the cytoplasm of WT macrophages whereas FABP4 deficiency reduced the expression of GPAT1, DGAT1 and lipidTOX in KO macrophages (**Figure 5G** [↗](#)). Quantitative analysis demonstrated that GPAT1, DGAT1 and lipidTOX levels were significantly reduced when FABP4 was absent (**Figure 5H** [↗](#)-**5J** [↗](#)). Using transmission electron microscopy, we further confirmed that FABP4 deficiency compromised LA- and OA-induced lipid accumulation in macrophages (**Figure 5L** [↗](#), Figure S5G). The role of FABP4 in lipid accumulation was corroborated by analysis of primary peritoneal macrophages from FABP4 WT and KO mice. As shown in Figure S5H and S5I, LA-induced neutral lipid accumulation in macrophages was significantly reduced when FABP4 was deficient. Collectively, these data suggest that FABP4 plays a pivotal role in TAG synthesis and neutral lipid accumulation in macrophages.

Macrophage expression of FABP4 promotes breast cancer migration

To further dissect the functionality of FABP4 in TAMs, we analyzed FABP4-associated gene pathways in the TCGA breast cancer datasets. Interestingly, FABP4 expression was highly associated with the lipolysis regulation pathway, including genes of β -adrenergic receptors

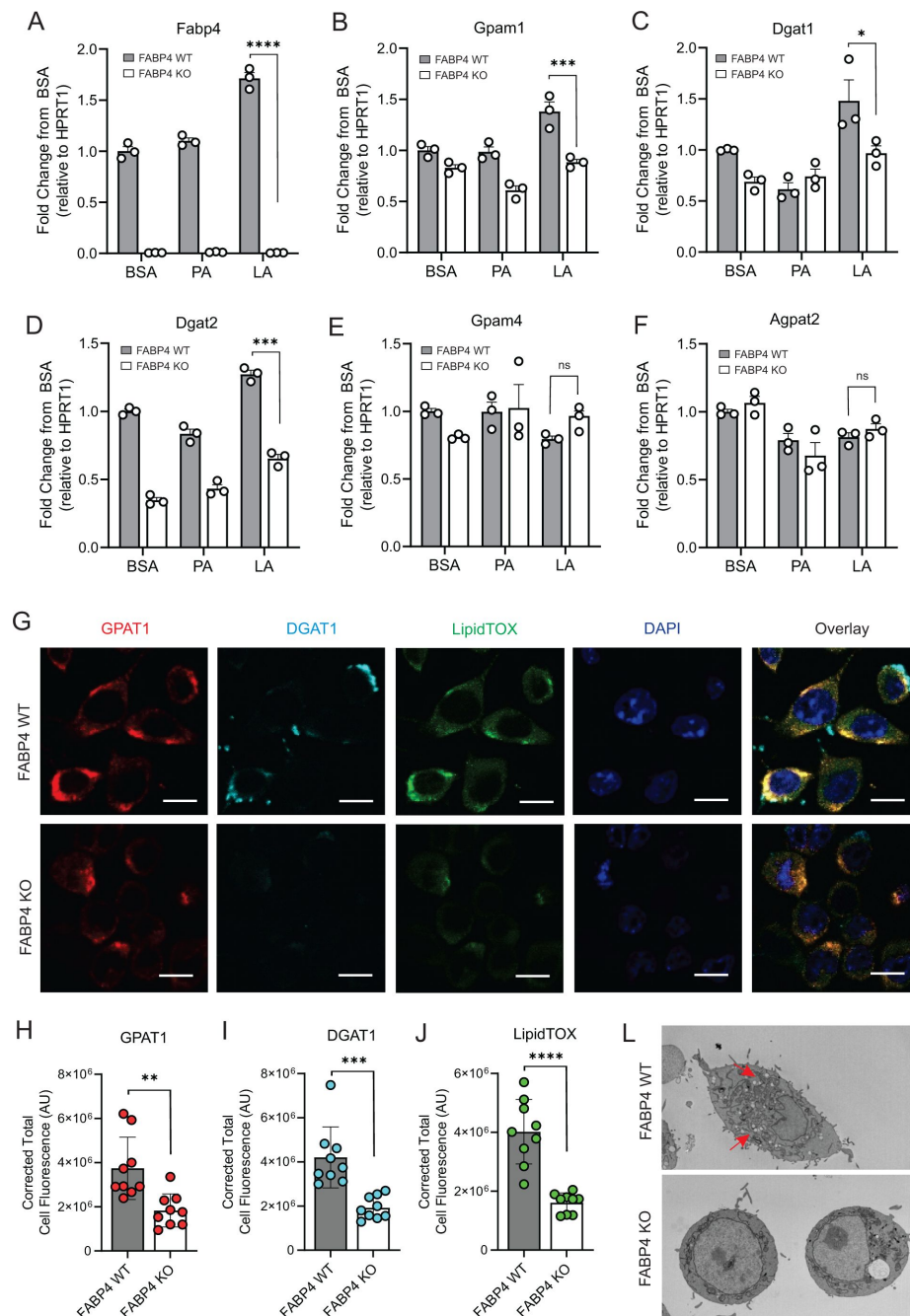


Figure 5

FABP4 deficiency reduces LA-induced lipid accumulation in macrophages

A-F, real-time PCR analysis of FABP4 (A) and genes encoding key enzymes for triglyceride biosynthesis, including *Gpm1* (B), *Dgat1* (C), *Dgat2* (D), *Gpm4* (E), *Agpat2* (F) in WT and FABP4 KO macrophages treated with BSA, PA or LA (400μM). G, Confocal analysis of protein expression of GPAT1 (red), DGAT1 (cyan) and lipid accumulation (LipidTOX staining, green) in LA-treated WT and FABP4 KO macrophages (bar, 10μM). H-J, Expression levels of GPAT1 (H), DGAT1 (I) and LipidTOX (J) as indicated in panel G were quantified by Image J. L, Flow cytometric analysis of neutral lipid accumulation as shown by BODIPY staining in WT and FABP4 KO macrophages treated with BSA or LA. M, Transmission electron microscope showing lipid droplet staining in WT and FABP4 KO macrophages treated with LA. Data are shown as mean ± SD in panel A-F, H-L (* $p \leq 0.01$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns, non-significant as compared to the control BSA group or FABP4^{-/-} group, unpaired Student t test). See also Figure S5.

(Adrb2), G-protein-coupled receptor signaling (Adcy4), and lipolysis enzymes (Pnpla2, Lipe) (Figure S6A-S6D). When FABP4 WT and KO macrophages were treated with PA or LA, FABP4 deficiency impaired LA-induced lipolysis in macrophages as evidenced by significantly reduced levels of Adrb2, Adcy4, Pnpla2 and Lipe (Figure 6A–6D), suggesting a critical role of FABP4 in mediating lipolysis-associated pathways in macrophages. Given the protumor role of lipid accumulation in TAMs, we treated FABP4 WT or KO macrophages with PA, LA or BSA, and evaluated their protumor function using trans-well tumor migration assays (Figure 6E). As shown in Figure 6F and 6G, compared to BSA- or PA-treated WT macrophages, LA-treated macrophages greatly promoted MDA-MB-231 cell migration. Notably, FABP4 deficiency significantly reduced LA-mediated tumor migration. Using E0771 mammary tumor cells, we confirmed the essential role of FABP4 in mediating LA-induced tumor migration effect in macrophages (Figure 6H, 6I). The FABP4/LA-induced tumor migration effect was not due to tumor proliferation as Ki67 expression was similar among different FA-treated groups (Figure S6E). Instead, lipid droplets formed by LA in WT macrophages were depleted following coculture with tumor cells (Figure 6J), showing the pro-migration effect of FABP4-mediated lipolysis. Furthermore, we collected the conditioned media collected from FA-treated macrophages and demonstrated that only media from LA-treated macrophages promoted migration of MDA-MB-231 and E0771 in a FABP4-dependent manner (Figure S6F-6I). These data suggested that intracellular FABP4 was secreted from macrophages into the extracellular environment to mediate the pro-migration effect. Indeed, LA-treated macrophages exhibited the highest levels of FABP4 compared to other groups (Figure S6J). Altogether, FABP4 expression in macrophages was essential for LA-induced pro-tumor effects.

High expression of FABP4 in TAMs is associated with breast cancer metastasis

Recently, meta-analysis demonstrated that a high density of TAMs, especially CD163⁺ TAMs, predicted poor survival outcomes in breast cancer⁴. When we analyzed pro-tumor activity of TAMs using human breast cancer tissue, we verified that high expression of the macrophage marker CD163 was significantly associated with a reduced overall survival of these breast cancer patients in multiple breast cancer datasets (Figure S7A-S7D). To verify the results from these publicly accessible datasets, we collected breast cancer tissue specimens from a cohort of 59 women with different subtypes of breast cancer (Table S1) and performed immunohistochemical (IHC) staining. Interestingly, CD163⁺ TAMs were positively correlated with breast tumor size (Figure 7A, 7B), corroborating the pro-tumor function of CD163⁺ macrophages in breast cancer. We also noticed that compared to lean patients, obese patients had more CD163⁺ macrophages in the TME (Figure S7E, S7F), suggesting that dysregulated lipids in obese patients promoted macrophage tumor infiltration. Given the pivotal role of FABP4 in lipid metabolism in macrophages, we analyzed FABP4 expression in CD163⁺ TAMs. Interestingly, FABP4 expression significantly correlated with CD163⁺ macrophages ($p < 0.001$) (Figure 7C). Among the cohort, 22% of patients exhibited lymph node and distant metastasis (Figure 7D). When we compared macrophages and FABP4 expression in tumor tissues in patients with or without tumor metastasis (Figure 7E), we showed that tumors from metastatic patients had higher expression of CD163⁺ macrophages (Figure 7F) and FABP4 expression (Figure 7G). We also examined the association of other variables, including age, tumor grade, multifocality, lymphovascular invasion, mortality, hormone status, with CD163 and FABP4 expression in tumor tissues. Higher tumor grade and mortality were associated with more FABP4 expression in macrophages (Figure 7H, Table S2). Moreover, triple negative breast cancer exhibited higher FABP4 expression in macrophages than ER⁺ or HER2⁺ breast cancer (Figure S7G, S7H). Collectively, CD163⁺ TAMs, especially those with FABP4 expression, are positively associated with tumor growth and metastasis in breast cancer patients.

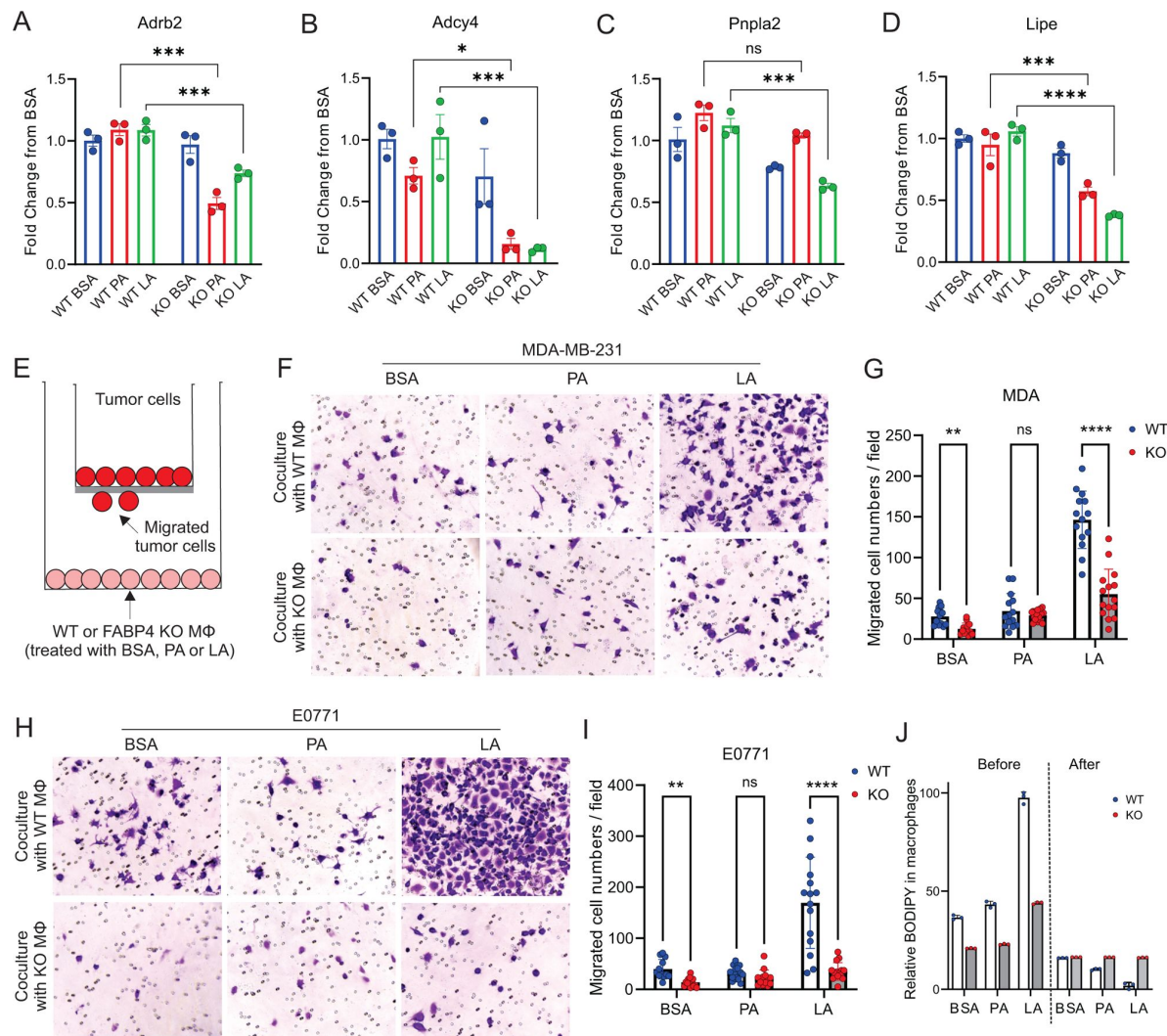


Figure 6

FABP4 expression in macrophages promotes lipolysis and breast cancer cell migration

A-D, Realtime PCR analysis of expression of *Adrb2* (A), *Adcy4* (B), *Pnpla2* (C) and *Lipe* (D) in FABP4 WT and KO macrophages treated with BSA, PA or LA (400μM).

E, Transwell measurement of migration of breast cancer cells cocultured with FA- or BSA-treated FABP4 WT or KO macrophages (Mφ).

F-I, FABP4 WT or KO macrophages were treated with 100μM BSA, PA or LA for 4 hours. Fatty acids in the culture medium were washed away with FBS-free RPMI-1640. Breast cancer cells were added to a transwell and cocultured with these different FA- or BSA-treated FABP4 WT or KO macrophages for 24 hours. The migrated tumor cells were stained and quantified. Migrated MDA-MB-231 cells were shown in panel F and G. Migrated E0771 cells were shown in panel H and I.

J, FABP4 WT and KO macrophages were treated with indicated FAs or BSA for 4 hours. Flow cytometric staining of BODIPY levels in WT and KO macrophages before and after coculture with E0771 tumor cells for 24 hours.

Data are shown as mean ± SD in panel A-D, G, I, J (* $p \leq 0.01$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns, non-significant as compared to the control group or FABP4 KO group, unpaired Student t test). See also Figure S6.

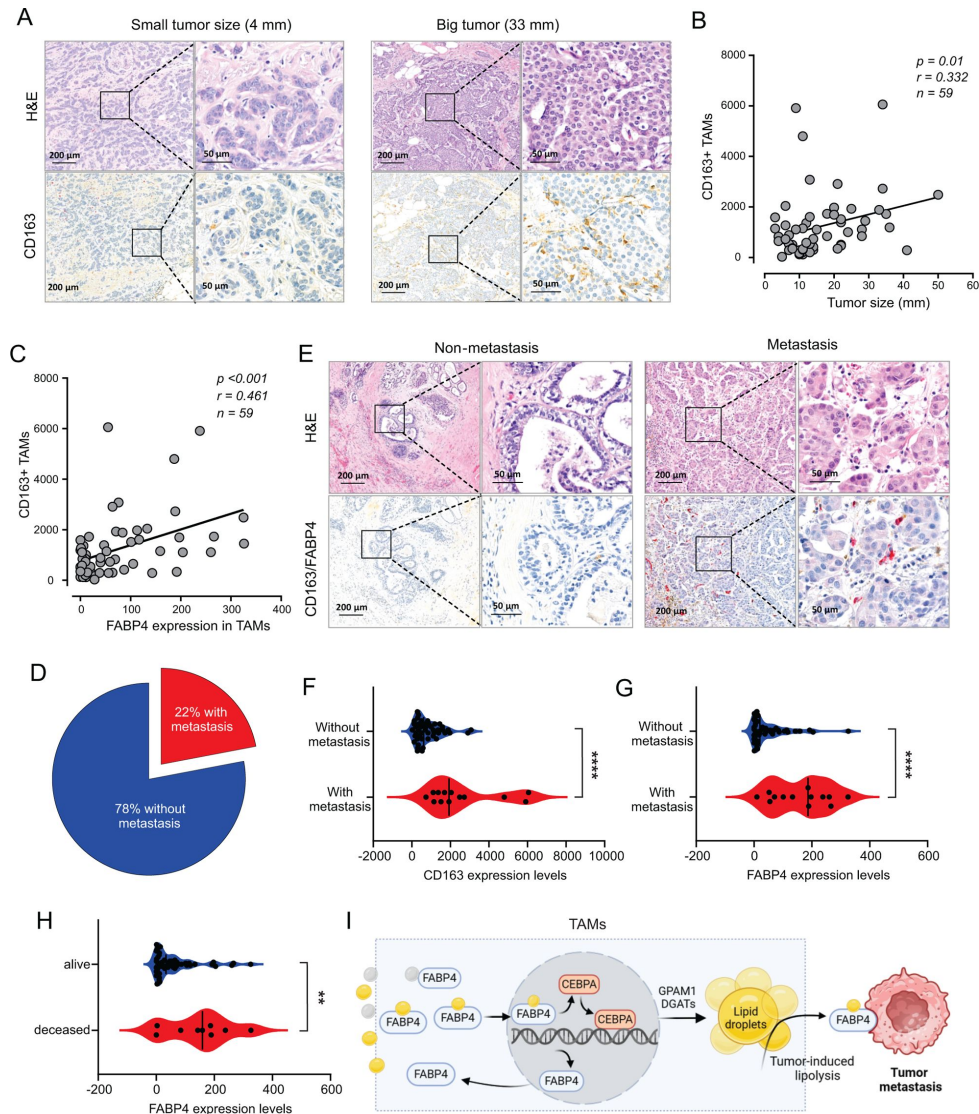


Figure 7

High expression of FABP4 in TAMs is associated with more metastasis of breast cancer

A, Comparison of H&E and CD163 staining (brown) between an example of small and large breast cancer tumors in breast cancer patients.

B, Spearman correlation analysis between breast cancer tumor size and CD163+ TAM staining. C, Expression of FABP4 and CD163 was highly correlated as analyzed by the Spearman correlation analysis in breast cancer tissues.

D, Pie chart showing the percentage of breast cancer patients with or without metastasis.

E, Analysis of the staining of H&E, CD163 (brown), FABP4 (red) in primary breast tumors of patients with and without metastasis.

F, Analysis of CD163 expression levels between primary breast tumors of patients with and without metastases.

G, Analysis of FABP4 expression levels between primary breast tumors of patients with and without metastases.

H, Analysis of FABP4 expression levels between alive and deceased breast cancer patients.

I, Scheme of how FABP4 mediates unsaturated FA (yellow)-induced lipid storage and lipolysis in TAMs. When TAMs are exposed to dietary saturated (gray) or unsaturated (yellow) FAs, unsaturated FAs, but not saturated ones, induce FABP4 nuclear translocation and upregulate FABP4 and CEBPA-mediated transactivation of GPAM1 and DGATs, promoting lipid storage as lipid droplets. Once tumor-induced lipolysis occurs, FABP4/unsaturated FAs are secreted from TAMs to induce tumor migration and metastasis.

Data are shown as mean $\pm$ SD in panel F-H ($p^{**} p \leq 0.01$, $^{***} p \leq 0.001$, $^{***} p \leq 0.001$, unpaired Student t test). See also Figure S7.

Discussion

Breast cancer is the most prevalent cancer in women globally ³⁶. The World Health Organization reported in 2020 that 2.3 million women were diagnosed with breast cancer, with 685,000 deaths from the disease ^{36,37}. Given that metastatic breast cancer is the leading cause of breast cancer-related death ³⁸, identifying new cellular and molecular mechanisms underlying breast cancer metastasis is an urgent need for breast cancer research. The TME of breast cancer is lipid enriched, and these lipids can be taken up by macrophages, leading to their alternative activation ³⁹. Alternative activated macrophages are known to stimulate angiogenesis and promote tumor progression ¹⁶. However, the molecular mechanisms by which extracellular lipids contribute to intracellular lipid accumulation and pro-tumor functions in macrophages are largely unexplored.

As professional phagocytes, macrophages are engaged with uptake of extracellular lipids to mitigate their toxicity and to process them for use by themselves or adjacent cells ⁴⁰. Macrophages have evolved to mainly express FABP4 and FABP5 to facilitate FA transport and metabolism. We previously demonstrated that heterogenous macrophages exhibit different FABP expression profiles and activities. For instance, FABP5 expression in skin macrophages facilitates high fat diet-induced IL-1 family cytokine responses (e.g., IL-1 β , IL-36 γ) while FABP4 expression in TAMs promotes oncogenic IL-6/STAT3 signaling ^{19,41,42}. We further demonstrated that heterogenous macrophages exhibit a unique FABP expression profile. FABP4 is prominently expressed in CD11b⁺F4/80⁺CD11c[−]MHCII[−] macrophage subsets whereas FABP5 is mainly expressed in CD11b⁺F4/80⁺MHCII⁺CD11c⁺ macrophage subsets ^{19,33,43}, suggesting a distinct role of individual FABP family members in macrophages ^{19,44}. Interestingly, when macrophages were exposed to different species of dietary FAs, FABP4 appeared to be more responsive to FA treatment. Notably, FABP4 was mainly located in the cytoplasm under PA treatment but translocated to the nucleus in response to LA treatment. This was consistent with previous studies showing LA as an activating ligand for FABP4 nuclear translocation and PPAR γ activation ⁴⁵. Considering that PPAR γ induces CEBP α expression by binding to its enhancer and they synergistically enhance lipid accumulation in adipocytes ^{46,47}, we demonstrated that LA treatment specifically upregulated CEBP α expression and induced lipid droplet formation in macrophages. Importantly, CEBP α transcriptionally upregulated key enzymes for TAG synthesis, including GPAT1 and DGATs. Moreover, silencing CEBP α expression significantly inhibited LA-induced GPAT1 and DGAT1 expression and lipid droplet accumulation. Therefore, our data suggest a previously underappreciated axis of LA/FABP4/CEBP α in mediating lipid accumulation in macrophages.

To determine the role of the accumulated lipids in macrophages, we noticed that FABP4 expression was not only associated with the LA/CEBP α /DGAT-mediated lipid accumulation pathway, but also highly correlated with the β -AR/ATGL/HSL-mediated lipolysis pathway in breast cancer. Genetic deletion of FABP4 inhibited both lipid accumulation and lipolysis pathways, confirming a critical role of FABP4 in lipid metabolism in macrophages. When breast cancer cells were cocultured with FA-exposed macrophages, LA-treated macrophages dramatically promoted breast cancer cell migration as compared to PA- or BSA-treated cells, suggesting a specific effect of LA-mediated lipid accumulation and utilization in macrophages. Moreover, FABP4 deficiency impaired LA-mediated protumor migration effect of macrophages, further supporting that FABP4-mediated lipolysis in macrophages was critical for lipid utilization by breast cancer cells.

TAMs are the most abundant immune cells in breast cancer. To interrogate TAM's role in the TME, M1/M2 dichotomy has been commonly proposed to polarize TAM functions. However, there have been no convincing markers or precise molecular mechanisms to determine TAM functions *in vivo*. By analyzing FABP4 expression in TAMs with human breast cancer specimens, we found that

FABP4 expression in TAMs, especially CD163⁺ TAMs, correlated directly with tumor size, grade, metastasis and even patient survival. Considering the critical role of FABP4 in mediating lipid accumulation and lipolysis, it might function as a key player linking extracellular lipids to lipid storage and utilization in TAMs. Of note, compared to lipid-processing macrophages, most epithelial cell-derived cancer cells do not express FABP4, thus FABP4 may serve as a functional marker for lipophilic pro-tumor TAMs.

It is worth noting that dietary fats are linked to both breast cancer incidence and survival in epidemiologic studies⁴⁸. However, the exact role of major dietary FAs, including PA, SA, OA, and LA, in breast cancer risk and progression remains unclear. Given the observations that saturated fat intake increases the risk of cardiovascular diseases, the American Dietary Guidelines recommend the consumption of unsaturated fats over saturated fats in our diets (<https://www.dietaryguidelines.gov/>). However, data from the present study raise the concerns about the consumption of unsaturated fats, especially LA, in patients with breast cancer. Unsaturated FAs can induce lipid accumulation in macrophages, which can subsequently be mobilized to facilitate breast cancer migration and metastasis. Furthermore, we demonstrated that FABP4 plays a critical role in facilitating unsaturated FA-induced storage and lipolysis. Upon activation of LA, FABP4 trafficks between the cytosol and nucleus to mediate lipid accumulation. In the presence of external factors (e.g., tumors), FABP4 can be secreted from macrophages during lipolysis, which in turn enhances the utilization of FA ligands by cancer cells. Thus, blocking FABP4 lipid binding and secretion might represent novel strategies for preventing lipid utilization by cancer cells.

In summary, our studies have several implications. First, as professional phagocytes, macrophages uptake different lipids. Unlike saturated FAs, unsaturated FAs are preferentially accumulated in macrophages through the FABP4/CEBPα pathway. Secondly, TAMs with lipid accumulation facilitate breast cancer progression through the FABP4-dependent lipolysis and lipid utilization pathways. Thirdly, secreted FABP4/FAs might offer a previously underappreciated mechanism for promoting breast tumor metastasis potential (**Figure 7I**). Thus, developing therapies that block the activity of the secreted FABP4 might provide a new avenue for the treatment of breast cancer.

Limitations of the study

In this study, we have identified FABP4 as a crucial lipid chaperone responsible for mediating lipid accumulation and lipolysis in TAMs. However, the mechanisms by which FABP4/FA complexes secreted from TAMs interact with tumor cells and mediate tumor metastasis remain unknown. While some potential FABP4 receptors, such as membrane phospholipids⁴⁹, desmoglein 2⁵⁰, cytokeratin 1⁵¹, have been reported in various cell types, further investigations are needed to determine how FABP4 and its lipid ligands facilitate tumor metastasis - either by initiating oncogenic signals, by providing energy sources or both.

Acknowledgements

We thank the Iowa Institute of Human Genetics at the University of Iowa for single cell RNA-sequencing and analysis. We also thank the Holden Comprehensive Cancer Center's P30 grant which partially funds the Breast Molecular Epidemiology Resource (BMER) (P30CA086862). B.L. thank the funding support from NIH grants U01CA272424, R01CA180986 and R01AI137324. This material should not be interpreted as representing the viewpoint of the U.S. Department of Health and Human Services, the National Institutes of Health, or the National Cancer Institute.

Author contributions

M.Y., X.J, S.L., J. H., J.Y., A.A. performed experiments, analyzed and discussed the data. J.H. did the single cell sequencing. lysis. Y.X., helped with reading the histological slides, H. K. did the single cell RNA-seq analysis. Z.L., and M.K and did the statistical analysis. Y. X, A.K., and M.C. provided human samples and helped with histological sections. X.J., and J.S. performed the transmission electron microscopy analysis. E. S., S. S. and B.L. designed experiments and wrote the paper.

Declaration interests

Authors declare no competing interests.

Methods

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
PE/Cyanine7 anti-mouse F4/80	BioLegend	Cat#123114
Brilliant Violet 711 anti-mouse CD11c	BioLegend	Cat#117349
Brilliant Violet 605 anti-mouse I-A/I-E	BioLegend	Cat#107639
BUV737 rat anti-CD11b	BD Biosciences	Cat#612800
BUV563 rat anti-mouse CD45	BD Biosciences	Cat#752412
Anti-hFABP4 goat polyclonal	R&D Systems	Cat#AF3150
Anti-m/rFABP4 goat polyclonal	R&D Systems	Cat#AF1443
GPAM rabbit polyclonal	Invitrogen	Cat#PA5-20524
Anti-DGAT1 goat polyclonal	Sigma-Aldrich	Cat#SAB2500307
Anti-C/EBP α rabbit polyclonal	Cell Signaling	Cat#2295
Anti-C/EBP β mouse monoclonal	DSHB	Cat#PCRP-CEBPB-3D10
Anti-CD163 human monoclonal	Abcam	Cat#EPR19518
Alexa Fluor 594-conjugated AffiniPure Mouse anti-goat IgG	Jackson ImmunoResearch	Cat#205-605-108
Alexa Fluor 647-conjugated AffiniPure Donkey anti-goat IgG	Jackson ImmunoResearch	Cat#705-586-147
Donkey anti-goat IgG Alexa Fluor Plus 488	Invitrogen	Cat#A32814
Alexa Fluor 647-conjugated AffiniPure Goat anti-rabbit IgG	Jackson ImmunoResearch	Cat#111-605-003
Goat anti-rabbit IgG Alexa Fluor 546	Invitrogen	Cat#A11035
Goat anti-mouse IgG Alexa Fluor 488	Invitrogen	Cat#A11029
Goat anti-mouse IgG Alexa Fluor 546	Invitrogen	Cat#A11030
Chemicals, Peptides, and Recombinant Proteins		
Ghost Dye Violet 510 Viability Dye	Tonbo Biosciences	Cat#13-0870-T100
Zombie Violet™ Fixable Viability Kit	Biolegend	Cat#423108
Bodipy 493/503	Molecular Probes	Cat#D3922
LysoTracker Blue DND-22	Invitrogen	Cat#L7525
Cell Navigator Live Cell Endoplasmic Reticulum Staining Kit Red	ATT Bioquest	Cat#22636
MitoSpy NIR DiIC1(5)	BioLegend	Cat#424807
HCS LipidTOX Green Neutral Lipid Stain	Invitrogen	Cat#H34475
Hoechst 33342 Solution	Invitrogen	Cat#62249
DAPI solution (1mg/mL)	Invitrogen	Cat#62248
Oil Red O powder	Alfa Aesar	Cat#A12989.14
Recombinant Mouse M-CSF	BioLegend	Cat#576406
Recombinant Mouse GM-CSF	BioLegend	Cat#576304
Phosphate Buffered Saline pH 7.4 (PBS)	Gibco	Cat#10010-023
Paraformaldehyde Solution 4% in PBS	Thermo Fisher Scientific	Cat#J19943.K2
Neutral Buffered Formalin Solution 10% in PBS	VWR	Cat#10790-714
Triton X-100	Sigma-Aldrich	Cat#X100-500ML

RPMI Medium 1640	Gibco	Cat#11875-093
Seahorse XF RPMI Medium pH7.4	Agilent Technologies	Cat#103576-100
Seahorse XF calibrant solution	Agilent Technologies	Cat#100840-000
Penicillin-Streptomycin	Thermo Fisher Scientific	Cat#15140122
Fetal Bovine Serum	R&D Systems	Cat#S11550
Goat Serum	Thermo Fisher Scientific	Cat#31873
Palmitate	Nu-Chek Prep, Inc	S-1109
Stearate	Nu-Chek Prep, Inc	S-1111
Oleate	Nu-Chek Prep, Inc	S-1120
Linoleate	Nu-Chek Prep, Inc	S-1127
Bovine Serum Albumin	Proliant	Cat# 69760
Poly-L-lysine	Sigma-Aldrich	Cat#P9155
RBC Lysis Buffer	Tonbo Biosciences	Cat#TNB-4300-L100
Chromium NextGEM Chip G Single Cell Kit	10 X Genomics	Cat#1000127
Dual Index Kit TT Set A	10 X Genomics	Cat#1000215
Chromium NextGEM Single Cell 3'Kit v3.1	10 X Genomics	Cat#1000269
Magnetic Separator	10 X Genomics	Cat#120250
Non-acetylated BSA	10 X Genomics	Cat#B9000S
Critical Commercial Assays		
Seahorse XF Cell Mito Stress Test Kit	Agilent Technologies	Cat#103015-100
Seahorse XF96 Cell Culture Plate	Agilent Technologies	Cat#101085-004
Seahorse XFe96 Extracellular flux assay kits	Agilent Technologies	Cat#102601-100
PureLink RNA mini kit	Invitrogen	Cat#12183025
QuantiTect reverse transcription kit	Qiagen	Cat#205314
ImmPRESS® HRP Horse Anti-Rabbit IgG Polymer Detection Kit, Peroxidase	Vector	Cat#MP-7401-50
BLOXALL® Endogenous Blocking Solution, Peroxidase and Alkaline Phosphatase	Vector	SP-6000-100
CircuLex Mouse FABP4/A-FABP ELISA Kit	CircuLex/MBL	Cat#CY-8077
Real-time PCR Primers	Table S1	N/A
DsiRNA for mouse C/EBP α	IDT	Cat#mm.Ri.Cebpa.13.1
DsiRNA for mouse C/EBP β	IDT	Cat#mm.Ri.Cebpb.13.1
jetPRIME	Sartorius	Cat#101000046
Power SYBR Green PCR Master Mix	Applied Biosystems	Cat#4368708
Deposited Data		
Microarray data (Breast Cancer Gene Expression Analysis)	PMID:23740839	GSE42568

Microarray data (Expression profiling of human DCIS and invasive ductal breast carcinoma)	PMID:21468687	GSE21422
Microarray data (Identifying breast cancer biomarkers)	PMID:141503	GSE29431
NKI	PROGeneV2	https://bioconductor.org/packages/breastCancerNKI
Microarray data	PROGeneV2	GSE10893-GPL887
Microarray data	PROGeneV2	GSE18229-GPL887
Microarray data	PROGeneV2	GSE9893
Experimental Models: Cell Lines		
Bone Marrow Derived Macrophage-J2 Immortalized	This Paper	N/A
FABP4 ^{-/-} Bone Marrow Derived Macrophage-J2 Immortalized	This Paper	N/A
BMDM	This paper	N/A
Peritoneal Macrophage	This paper	N/A
E0771	ATCC	Cat#CRL-3461
MDA-MB 231	ATCC	Cat#HTB-26
Experimental Models: Organisms/Strains		
Mouse:C57Bl/6J	Jackson Laboratory	JAX 000664
Mouse:FABP4 ^{-/-}	This Paper	
Oligonucleotides		
Real-time PCR Primers	Table S1	N/A
Software and Algorithms		
FlowJo v10	BD Biosciences	www.flowjo.com
GraphPad Prism 8	GraphPad Software	www.graphpad.com
ImageJ (Fiji edition)	NIH	https://imagej.net/software/fiji/
Design and Analysis Software V2.3	Thermo Fisher	http://www.thermofisher.com/us/en/home/global/forms/life-science/quantstudio-6-7-pro-software.html
Seahorse Wave	Agilent Technologies	https://www.agilent.com/zh-cn/product/cell-analysis/real-time-cell-metabolic-analysis/xf-software/seahorse-wave-desktop-software-740897
Web-based Gene Set Analysis Toolkit		https://www.webgestalt.org/
VolcanoR2		https://huygens.science.uva.nl/VolcanoR2/
g:Profiler		https://biit.cs.ut.ee/gprofiler/gost
CiiiDER		https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0215495
CoreIDRAW X7	CoreIDRAW	www.coreldraw.com
Halo Software	Indica Labs	V3.6.4134.263
Other		
Sterile 24 well cell culture plate	Corning	Cat#3526
Sterile 100mm cell culture dish	Greiner Bio-One	Cat#664160

Sterile 25G x 1" Needle	Becton, Dickinson and Company	Cat#305125
Sterile 10mL syringe	Becton, Dickinson and Company	Cat#302995
Sterile 15mL conical-bottom Centrifuge Tube	Avantor	Cat#525-1068
40µm cell strainer	VWR	Cat#BX15-1040
Transwell inserts 8.0µm pore size	Falcon	Cat#353097
5mL Polystyrene round-bottom tube	Falcon	Cat#352058
MicroAmp Optical 384-well Reaction Plate	Applied Biosystems	Cat#4309849
QuantStudio 7 Flex Real-Time PCR system	Applied Biosystems	Cat#4485701
Seahorse XFe96 extracellular flux analyzer	Seahorse Biosciences	
Cytek Aurora	CytekBio	
Cytek Amnis ImageStream MkII Imaging Flow Cytometer	CytekBio	
Synergy LX Multi-Mode Microplate Reader	BioTek	Cat#SLXFATS-SN
Echo Revolution 2 microscope	ECHO	
Zeiss LSM880-airyscan	Zeiss	
Electron Microscope	Hitachi	HT7800
Slide Scanner	Leica	Aperio GT 450

Resource availability

Lead contact

Further information and requests for reagents may be directed to and will be fulfilled by the Lead Contact, Bing Li (bing-li@uiowa.edu).

Materials availability

All unique/stable reagents generated in this study are available from the lead contact with a completed materials transfer agreement.

Data and code availability

All data generated in this study will be made available upon request from the Lead Contact. This paper reports single cell sequencing data (GSE262196). Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental model and study participant details

Mice

Fabp4^{-/-} mice and their wild-type (WT) mice (C57B/6 background) were maintained and bred under specific pathogen free conditions with water and regular chow ad libitum in the animal facility of the University of Iowa in accordance with the approved protocol by the University of Iowa Institutional Animal Care and Use Committee (#1042385). As breast cancer mainly occurs in female, female mice at 8-12 weeks age were used for collection of primary peritoneal macrophages, splenic macrophages and culture of bone marrow-derived macrophages.

Patient specimens

Fresh or formalin-fixed, paraffin-embedded breast cancer tissues were collected from the Breast and Molecular Epidemiology Resources (BMER) or Department of Pathology, University of Iowa. Breast sections were cut and transported to the study team without further patient identifiers. The University of Iowa Institutional Review Board approved the study protocol (#201003791, 202107133) prior to sample acquisition.

Method details

Primary Cells

WT and age matched FABP4^{-/-} mice were euthanized by CO₂ inhalation and death was confirmed via subsequent cardiac puncture. Peritoneal macrophages were isolated by injecting 10mL PBS (Gibco) into the mouse peritoneal cavity and incubated with 3 minutes with periodic abdominal palpation. EtOH was used to sterilize the surgical site and the skin of the abdomen was reflected. Using a 10mL syringe coupled with a 25G needle (Becton Dickinson and Company), PBS from the peritoneal cavity was aspirated, moved to a 15mL conical (Avantor) tube, centrifuged, and resuspended in RPMI media with 10% FBS. To obtain bone marrow derived macrophages (BMM) femurs were dissected, muscle tissue was thoroughly removed, and marrow was flushed with PBS into a 15mL conical tube by way of a 10mL syringe coupled with a 25G needle. Isolated marrow cells were resuspended in red blood cell lysis buffer (Tonbo Biosciences) for 15 minutes on ice, washed with PBS, filtered through 40µm cell strainers (VWR) and plated on 100mm culture dishes (Greiner Bio-One) at a concentration of 5×10⁶ cells/mL in RPMI media with 10% FBS and 30ng/mL recombinant mouse macrophage colony-stimulating factor (M-CSF; BioLegend). After 48 hours and again after 120 hours from initial plating cell culture media was replaced with fresh media containing M-CSF. Cells were scrutinized after 168 hours in culture.

Cell Lines

Immortalized macrophage cell lines were established from FABP4^{-/-} (FABP4^{-/-} macrophages) or WT mice (WT macrophages) as described previously^{33,52,53}. Briefly, bone marrow derived macrophages were culture as outlined above however following differentiation J2-virus conditioned media was used for 2 hours and replaced with M-CSF media. This procedure was repeated after 24 hours. Subsequently M-CSF media was removed step-wise over the course of 2 weeks at which point cells could be effectively passages. This procedure was completed for marrow cells obtained from a C57Bl/6J mouse as well as aged match FABP4^{-/-} mouse. Breast cancer cell migration assays were completed using E0771 mouse mammary gland cells (CH3 Biosystems) and MDA-MB 231 human mammary gland cells (ATCC).

Free fatty acid (FFA) preparation

Due to their insolubility, all FFAs were conjugated with bovine serum albumin (BSA) as described previously^{33,44,54,55}. Briefly, all FFAs used in the current study were purchased from Nu-Chek Prep (MN). PA (5 mM), SA (5 mM), OA (5 mM) or LA (5 mM) were respectively conjugated with 2 mM of endotoxin-free, FA-free BSA in PBS. FA-BSA conjugates were sonicated until dissolved. All FA-BSA conjugates or BSA control were filtered through a 0.2-µM sterile filter before use in cellular studies.

RNAi

Predesigned duplex small interfering RNA was obtained from Integrated DNA Technologies (IDT) specific to C/EBPα and C/EBPβ. J2-Immortalized macrophages were transfected using jetPRIME (Polyplus) as instructed by the manufacture. Efficacy of RNAi was confirmed by RT-PCR for each

experiment.

Quantitative RT-PCR

Cells were lysed and RNA was extracted using the PureLink RNA mini kit (Invitrogen) as per manufactures instructions. RNA concentration was determined and standardized by the aid of a photospectrometer and complementary DNA was synthesized by use of QuantiTect reverse transcription kit (Qiagen). Power SYBR Green PCR Master Mix (Applied Biosystems) was included with target primers (Table S3) on MicroAmp optical 384-well reaction plates (Applied Biosystems) and a QuantStudio 7 Flex (Thermo Fisher) and the provided software (Design and Analysis Software V2.3, Thermo Fisher) was used to determine relative mRNA concentration by way of $\Delta\Delta C_t$ method with *hprt1* as an internal control. Standard PCR reaction parameters were used: 95°C hold stage for 10 minutes, 40 cycles of PCR reaction of 95°C for 15 seconds and 60°C for one minute, and a melt curve stage used 0.5°C steps from 60°C to 65°C.

Seahorse Cell Mito Stress

The Seahorse mitochondrial stress test (Agilent) was performed as specified by the manufacturer. Briefly, cells were plated at a concentration of 5×10^4 per well in RPMI media with 10% FBS. Before the assay cells were wash twice with RPMI-XF media with no FBS and incubated at 37°C for 15 minutes during equipment initialization. supplemented with 100μM BSA, PA, SA, OA, or LA. The acute injection protocol was utilized where some wells (n=5 for each FA for each genotype) received injections of RPMI-XF supplemented with 200μM BSA, PA, SA, OA, or LA, and all wells subsequently received injections of 2.5μM oligomycin, 1μM FCCP, and finally 1μM Rotenone/Antimycin/Hoechst. Assays were ran using the XFe96 extracellular flux analyzer (Agilent) coupled with automated normalization based on live cell number at the conclusion of the assay.

Breast Cancer Tissue immunohistochemistry

Formalin fixed Paraffin Embedded (FFPE) tissue specimens were deparaffinized in xylene and rehydrated through exposure to graded ethanol solutions. Antigen retrieval was performed by incubating the slides in 10 mM Citrate buffer pH6.0 at 95°C for 15 min. Then, quenching of endogenous peroxidase activity was performed by incubating the slides in BLOXALL Endogenous Blocking Solution (Vector Laboratories, SP-6000) for 10 min at room temperature. Slides were incubated with a primary antibody overnight at 4 °C, a secondary antibody for 30 min at room temperature (All the primary and secondary antibodies used in this study are summarized in key resources table). DAB peroxidase substrate (Vector Laboratories, SK-4100) or Vector Red alkaline phosphatase substrate (Vector Laboratories, SK-5100) were used for staining. Counterstain was performed by incubating the slides in Hematoxylin (Leica Gill III 3801541) for 10 seconds at room temperature. Slides were mounted with VectaMount® Express Mounting Medium (vector laboratories, H-5700-60), and were scanned by slide scanner (Leica Aperio GT 450) for quantification analysis. Hot spot quantification of CD163 and FABP4 expression in macrophages (per $1 \times 2 \text{ mm}^2$ area within the tumor tissue) was automatically calculated by the HALO software (Indica Labs).

Transmission Electron Microscopy

Cells were fixed with 2.5% glutaraldehyde (in 0.1 M Sodium cacodylate buffer [pH 7.4]) overnight at 4°C. samples were postfixied with 1% Osmium tetroxide for 1h and then rinsed in 0.1M Sodium cacodylate buffer. Following serial alcohol dehydration (50%, 75%, 95%, and 100%), the samples were embedded in Epon 12 (Ted Pella, Redding, CA). Ultramicrotomy was performed, and ultrathin sections (70 nm) were poststained with uranyl acetate and lead citrate. Samples were examined with a Hitachi HT-7800 transmission electron microscope (TEM) (Tokyo, Japan).

Immunogold analysis for FABP4 in macrophages

Cells were fixed with 4% paraformaldehyde (PFA), and then were dehydrated in graded concentrations of ethanol and embedded in LR White resin (Electron Microscopy Sciences, 14380). Ultrathin sections (70 nm) on grids were incubated with blocking solution (Aurion, 905.003) for 1 hr, and then incubated with primary antibody overnight at 4 °C, and then incubated with secondary antibody (Aurion, Rabbit-anti-Goat IgG (H&L) Ultra Small) for 2 hrs. Grids were re-fixed with 1% glutaraldehyde for 15 min and were incubated in silver enhancement (Aurion, 500.033) for 25 min. Sections were then stained with uranyl acetate and lead citrate and examined with a Hitachi HT-7800 transmission electron microscope (TEM) (Tokyo, Japan).

Oil Red O staining

For each use Oil Red O powder (Alfa Aesar) was solubilized in 100% isopropanol (75mg in 25mL) and mixed on a orbital shaker for 30 minutes to make a stock solution. For a working solution Oil Red O stock solution was used at a ratio of 3 parts Oil Red O to 2 parts ddH₂O and mixed on an orbital shaker for 10 minutes. The working solution was filtered twice through 0.2 µm syringe filter and used immediately on 4% PFA fixed cells. During the second of 3 washes with ddH₂O, DAPI was included at 1:5000 for nuclear identification. Images were taken with an ECHO Revolution2 (ECHO) epifluorescence microscope and processed using ImageJ/Fiji. Following image collection, for the purpose of semi-quantitative analysis, samples were washed with 60% isopropanol 3 times for 5 minute each and the residual Oil Red O was solubilized with 100% Isopropanol and absorbance was assessed by spectrophotometer at 492nm, 100% isopropanol was used as a background.

Flow Cytometry

The following anti-mouse conjugated antibodies were used to label macrophages surface receptors from peritoneal and bone marrow derived cells: PE/Cyanine7-F4/80, BV711-CD11c, BV605-I-A/I-E (BioLegend), BUV737-CD11b, and BUV563-CD45 (BD Biosciences). Unconjugated antibodies were used for quantification of intracellular markers: m/rFABP4 (R&D Systems), GPAM (Invitrogen), DGAT1 (Sigma-Aldrich), C/EBPα (Cell Signaling), C/EBPβ (DSHB). The following organelle dyes were used: BODIPY 493/503 (Lipid Droplets; Molecular Probes), Cell Navigator Live Cell Endoplasmic Reticulum Staining Kit Red (ATT Bioquest), and LysoTracker Blue DND-22 (Invitrogen). Dead cells were identified and removed from analysis via Ghost Dye Violet 510 (Tonbo Biosciences). Briefly, live cells were scrutinized unless fixation was required for intracellular targets in which case 4% PFA was used for 30 minutes to fix the cells and 0.2% Triton X-100 in PBS was used for permeabilization. Antibodies or organelle dyes were incubated with cells for 30 minutes on ice in the dark. When necessary secondary antibodies were incubated with cells for 1 hour on ice in the dark. Data was acquired using a Cytex Aurora 5-laser flow cytometer or ImageStream cytometer (CytexBio) and subsequently analyzed FlowJo V10 (BD Biosciences).

Migration Assay

For the migration assay, we treated WT and FABP4 KO macrophages with BSA, PA, or LA (100µM) for 4 hours in a 24-well plate. After washing them three times with PBS, we seeded E0771 or MDA-MB 231 cells (5 × 10⁴) into transwell inserts with a pore size of 8 µm (Corning). These cells were incubated with the FA-treated macrophages, respectively, to allow tumor cells to migrate. After a 24-hour culture at 37 °C, we removed the tumor cells inside the transwells that had not migrated using cotton swabs. The tumor cells that had migrated to the outside of the insert membrane were fixed with 4% PFA for 20 minutes and then stained with 0.1% crystal violet for 10 minutes. We counted the numbers of migrated E0771 and MDA-MB 231 cells in 12 random high fields under a

microscope. Each assay was performed in triplicate wells. Additionally, in some experiments, we used conditional media (RPMI-1640 without FBS) collected from BSA or FA-treated WT and KO macrophages to coculture with tumor cells and observe medium-induced tumor cell migration.

Single cell RNA-seq assay

Macrophages from mouse spleen were used for the 10 x Genomics 3' expression single cell assay. Briefly, splenocytes were isolated from two pairs of age-matched WT and FABP4^{-/-} female mice. Dead cells were excluded using Zombie-Violet and anti-CD16/32 antibody was used to block Fc receptors before additional staining with Alexa Fluor 488-conjugated anti-mouse F4/480 antibody and APC-conjugated anti-mouse CD11b antibody. The F4/80⁺/CD11b⁺/Zombie-Violet⁻ cells were sorted using a FACS Aira III instrument and resuspended at a concentration of 1000 cell/ μ L cold PBS with 0.04% non-acetylated BSA. An equal number of 5000 targeted cells from each sample were prepared to create GEMs. The GEM generation/barcoding, post GEM-reverse transcription cDNA amplification, and library construction were performed according to the manufacturer's guidelines.

Single cell data analysis

Single cell gene counting was performed by Cell Ranger (10X Genomics, version 7.1.0) using the refdata-gex-mm10-2020-A reference transcriptome. The resulting data matrices were subsequently imported into R (version 4.2.3) and analyzed using the Seurat package (version 4.9.9). Cells with less than 200 features or with percent mitochondrial gene expression greater than 5% were excluded from the analysis. The data across samples was integrated using the `IntegrateData` function. Gene expression was normalized and scaled using the default parameters. Based on visual inspection of the elbow plot, the first 30 Principal Components (PC) were used in UMAP-based dimensional reduction. The `FindClusters` function, with a resolution of 0.5, was then used to assign cells to clusters. The `FindMarkers` function was used to identify genes differentially expressed between wild-type and knockout samples or between different clusters within the same sample type. Feature plots and violin plots were used to visualize the results.

ELISA for FABP4 analysis

FABP4 excreted from cultured cells were analyzed with CircuLex Mouse FABP4 ELISA Kit (Medical and Biological Laboratories) as instructed by the manufactures. Briefly, for the ELISA 100 μ L of conditioned media was incubated in a 96-well plate precoated with FABP4 antibody, washed four times, incubated with the supplied secondary antibody, washed, and incubated with the developer to finally analyze with a spectrophotometer using a ratio of the absorbance at 450nm and 550nm.

Quantification and statistical analysis

All data were presented as the mean $\pm$ SD unless notified specifically. All experiment as indicated were performed by at least three independent experiments or technical replicates. For in vitro experiments, a two-tailed, unpaired student t-test, two-way ANOVA followed by Bonferroni's multiple comparison test, were performed by GraphPad Prism 9. For in vivo experiments analyzing associations of FABP4, TAMs and other factors in breast cancer patients, the Kruskal-Wallis test was applied to compare whether the outcome variable is significantly different across the different levels of a categorical predictor variable. Multiple linear regression models were employed to examine the association between the predictor variables and the outcome, where log transformation and Box Cox transformation were used to meet the underlying model assumption for normality. All statistical analyses were carried out in the statistical analysis software R. A statistics test is claimed to be significant if the p-value is less than 0.05.

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Editors

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Reviewer #1 (Public review):

Summary:

In the manuscript "FABP4-mediated lipid accumulation and lipolysis in tumor-associated macrophages promote breast cancer metastasis", Yorek, et al. provide a novel mechanism explaining how unsaturated fatty acids induce macrophages to accumulate lipid droplets, which when contained in tumor-associated macrophages (TAMs) are associated with increased metastasis in breast cancer. The authors conclude that unsaturated fatty acids are transported into macrophages by the chaperone FABP4 where they induce C/EBPalpha expression and transcriptional activity resulting in upregulation of enzymes involved in triacylglycerol and lipid droplet biosynthesis. The resulting accumulation of lipid droplets in macrophages creates a store of fatty acids that can subsequently be released through FABP4-dependent lipolysis and thereby stimulate the migration of nearby breast cancer cells. While generally well-written and developed, there are a few concerns about the rigor of experimental evidence that supports some conclusions, including the existence of a FABP4-C/EBPalpha pathway. Overall, the mechanism identified is a valuable contribution to our understanding of how tumor-associated macrophages may be influenced by available metabolites to promote the aggressiveness of certain cancers. FABP4 has the potential to be used as a novel biomarker of macrophage-induced cancer aggressiveness and/or a therapeutic target to prevent metastasis.

Strengths:

- (1) The study is logically organized and provides extensive evidence in support of the overall model proposed.
- (2) Multiple complementary techniques are used to identify and quantify lipid droplets.
- (3) Primary macrophages and macrophage cell lines are used and provide consistent data.
- (4) Knock-down and knock-out cells are used to assess the contributions of FABP4 and C/EBPalpha to gene expression.
- (5) Public gene expression data (GEO, TCGA) is used effectively throughout.

Weaknesses:

(1) After Figure 1, a single saturated (palmitic acid; PA) and a single unsaturated (linoleic acid; LA) fatty acid are used for the remaining studies, bringing into question whether effects are in fact the result of a difference in saturation vs. other potential differences.

(2) While primary macrophages are used in several mechanistic studies, tumor-associated macrophages (TAMs) are not used. Rather, correlative evidence is provided to connect mechanistic studies in macrophage cell lines and primary macrophages to TAMs.

(3) C/EBPalpha and FABP4 clearly regulate LA-induced changes in gene expression. However, whether these two key proteins act in parallel or as a pathway is not resolved by presented data.

(4) It is very interesting that FABP4 regulates both lipid droplet formation and lipolysis, yet is unclear if the regulation of lipolysis is direct or if the accumulation of lipid droplets - likely plus some other signal(s) - induces upregulation of lipolysis genes.

(5) In several places increased expression of genes coding for enzymes with known functions in lipid biology is conflated with an increase in the lipid biology process the enzymes mediate. Additional evidence would be needed to show these processes are in fact increased in a manner dependent on increased enzyme expression.

<https://doi.org/10.7554/eLife.101221.1.sa3>

Reviewer #2 (Public review):

The manuscript by Yorek et al explores the role of fatty acids, particularly unsaturated fatty acids, in lipid droplet accumulation and lipolysis in tumor-associated macrophages (TAMs). Using flow cytometry, immunofluorescent imaging, and TEM, the authors observed that unsaturated fatty acids, such as linoleic acids (LA), tend to induce lipid droplet accumulation in the ER of macrophages, but not in the lysosomes. This phenomenon led them to examine the key enzymes involved in lipid droplet/TAG biosynthesis, where they found incubation of LA upregulates GPAT/DGAT and C/EBPα. In vitro studies, data from public databases, single-cell RNA sequencing of splenic macrophages, and more show that FABP4 emerges as an important mediator for C/EBPα activation. This is further confirmed by FABP4-knockout macrophages, where lipid accumulation and utilization of unsaturated fatty acids were compromised in macrophages through inhibition of LA-induced lipolysis. Using the co-culture system and immunohistochemical analysis, they found that the high FABP4 expression in TAMs, which are observed in metastatic breast cancer tissue, promotes breast cancer cell migration in vitro.

This study is important since the impact of tumor microenvironment is crucial for the development of breast cancer. The individual experiments are well-designed and structured. However, the logic connecting to the next step is a bit difficult to follow, especially when combined with incomplete statistical analysis in some figures, making the conclusion less convincing. For instance, the comparison of macrophage FABP4 expression between breast cancer patients with or without metastasis illustrates the importance of FABP4 expression in metastasis, yet there is no examination of the expression of other key enzymes in the lipolysis or lipid biosynthesis pathway nor there is any correlation with parameters that would reflect patients' consumption of fatty acids. Similarly, an in vivo study comparing FABP4 knockout mice with or without unsaturated fatty acids would yield more compelling evidence. The statistical analysis was largely focused on the sets of unsaturated fatty acids when data from both types of fatty acids were present. In some cases, significant changes are observed in the sets of saturated fat, but there is no explanation of why only the data from unsaturated fats are important for investigation.

Overall, there is solid evidence for the importance of FABP4 expression in TAMs on metastatic breast cancer as well as lipid accumulation by LA in the ER of macrophages. A stronger rationale for the exclusive contribution of unsaturated fatty acids to the utilization of TAMs in breast cancer and a more detailed description and statistical analysis of data will strengthen the findings and resulting claims.

<https://doi.org/10.7554/eLife.101221.1.sa2>

Reviewer #3 (Public review):

Summary:

Regulated metabolism has only recently been recognized as a key component of cancer biology, and even more recently recognized as a significant modulator of the tumor microenvironment (TME). TAMs in the TME play a major role in supporting cancer cell survival and growth/spread, as well as generating an immunosuppressive ME to suppress anti-tumor immunity. Specific regulation of lipid metabolism in this context, in particular how lipids are stored and subsequently mobilized for metabolism, is largely unexplored - especially in the immunological components of the TME.

In this manuscript, the authors build on their previous observations that the fatty acid-binding protein FABP4 plays an important role in macrophage function and that FABP4 expression in tumor associated macrophages (TAM) promotes breast cancer progression. They demonstrate:

- (1) Unlike saturated fatty acids (FA), unsaturated FA promotes lipid droplet (LD) accumulation in murine macrophages. LD is the primary intracellular storage depot for FA.
- (2) Unsaturated FA activates the FABP4-C/EBPalpha axis to upregulate transcription of the enzymes involved in the synthesis of neutral triacylglycerol (TAG) is an essential step in the formation of the neutral lipid core of LD. It should be noted that the authors speculate that UFA-activated FABP4 translocates to the nucleus to activate PPARGgamma, which is known to induce C/EBPalpha expression, but do not directly test the involvement of PPARGgamma in this axis.
- (3) FABP4 deficiency compromises unsaturated FA-mediated lipid accumulation and utilization in murine macrophages.
- (4) FABP4-mediated lipid metabolism in macrophages (TAMS) contributes to breast cancer metastasis, in in-vitro of tumor migration induced by murine macrophages and in correlative studies from human patient breast cancer biopsies.

From these studies, the manuscript concludes that FABP4 plays a pivotal role in mediating lipid droplet formation and lipolysis in TAM, which provides lipids to breast cancer cells that contribute to their growth and metastasis.

These are significant findings, as they provide new insight into the mechanistic regulation of TAM biology via regulation of lipid metabolism, as well as define new biomarkers and potential novel therapeutic targets.

The findings are strong in the studies that mechanistically define the role of FAB4 in lipid accumulation and utilization in murine macrophages. However, evidence is less compelling regarding TAM biology and human breast cancer in 3 main areas:

First, while there is clear in vitro evidence that co-cultured murine macrophages genetically deficient in FABP4 (or their conditioned media) do not enhance breast cancer cell motility

and invasion, these macrophages are not bonafide TAM - which may have different biology. The use of actual TAM in these experiments would be more compelling. Perhaps more importantly, there is no in vivo data in tumor-bearing mice that macrophage deficiency of FABP4 affects tumor growth or metastasis - which are doable experiments given the availability of the FABP4 KO mice.

Second, no data is presented that the mechanisms/biology that are elegantly demonstrated in the murine macrophages also occur in human macrophages - which would be foundational to translating these findings into human breast cancer. It seems like straightforward in vitro studies in human monocytes/macrophages could be done to recapitulate the main characteristics seen in the murine macrophages.

Third, while the data from the human breast cancer specimens is very intriguing, it is difficult to ascertain how accurate IHC is in determining that the CD163+ cells (TAM) are in fact the same cells expressing FABP4 - which is the central premise of these studies. Demonstrating that IHC has the resolution to do this would be important. Additionally, the in vitro characterization of FABP4 expression in human macrophages would also add strength to these findings.

In summary, the strengths of this manuscript are the significance of metabolic regulation of the immune tumor microenvironment (TME), and the careful mechanistic delineation of FABP4 involvement in mediating lipid droplet formation and lipolysis in murine macrophages. The weaknesses of the work are the lack of direct experimental evidence that human macrophages behave in the same way as murine macrophages, the incomplete characterization of the role of FABP4 expression in TAM in modulating tumor growth in vivo (in murine models), and whether it can be definitively determined that FABP4 is being primarily expressed in the CD163+ macrophages in human breast cancer samples.

Strengths:

(1) Regulated metabolism has only recently been recognized as a key component of cancer biology, and even more recently recognized as a significant modulator of the tumor microenvironment (TME). TAMs in the TME play a major role in supporting cancer cell survival and growth/spread, as well as generating an immunosuppressive ME to suppress anti-tumor immunity.

(2) Regulation of lipid metabolism in this context is largely unexplored, especially in the immunological components of the TME.

(3) The work builds on the authors' previous work on the role of FABP4 plays an important role in macrophage function including FABP4 expression in TAM promotes breast cancer progression (Hao et al, Cancer Res 2018). This paper identified FABP4-expressing macrophages as being pro-tumorigenic via upregulation of IL-6/STAT3 signaling.

(4) The careful and thorough mechanistic delineation of FABP4 involvement in mediating lipid droplet formation and lipolysis in murine macrophages.

(5) The intriguing observations that FABP4-mediated lipid metabolism in macrophages contributes to breast cancer metastasis, in in vitro of tumor migration induced by murine macrophages and in correlative studies from human patient breast cancer biopsies that CD163+ cell numbers (putatively TAM) and FABP4 expression was associated with increased metastatic disease and poor overall survival.

(6) Identification of FABP4 both a prognostic biomarker and a potential therapeutic target to modulate the pro-tumor immune TME.

Weaknesses:

(1) While the authors speculate that UFA-activated FABP4 translocates to the nucleus to activate PPARgamma, which is known to induce C/EBPalpha expression, they do not directly test involvement of PPARgamma in this axis.

(2) While there is clear in vitro evidence that co-cultured murine macrophages genetically deficient in FABP4 (or their conditioned media) do not enhance breast cancer cell motility and invasion, these macrophages are not bonafide TAM - which may have different biology. Use of actual TAM in these experiments would be more compelling. Perhaps more importantly, there is no in vivo data in tumor bearing mice that macrophage-deficiency of FABP4 affects tumor growth or metastasis.

(3) Related to this, the authors find FABP4 in the media and propose that macrophage secreted FABP4 is mediating the tumor migration - but don't do antibody neutralizing experiments to directly demonstrate this.

(4) No data is presented that the mechanisms/biology that are elegantly demonstrated in the murine macrophages also occurs in human macrophages - which would be foundational to translating these findings into human breast cancer.

(5) While the data from the human breast cancer specimens is very intriguing, it is difficult to ascertain how accurate IHC is in determining that the CD163+ cells (TAM) are in fact the same cells expressing FABP4 - which is central premise of these studies. Demonstration that IHC has the resolution to do this would be important. Additionally, the in vitro characterization of FABP4 expression in human macrophages would also add strength to these findings.

<https://doi.org/10.7554/eLife.101221.1.sa1>

Author response:

Reviewer #1:

(1) After Figure 1, a single saturated (palmitic acid; PA) and a single unsaturated (linoleic acid; LA) fatty acid are used for the remaining studies, bringing into question whether effects are in fact the result of a difference in saturation vs. other potential differences.

PA, SA, OA and LA are the most common FA species in humans (Figure 1A in manuscript). Among them, PA predominantly represents saturated FAs while LA is the main unsaturated FAs, respectively. Of note, although both SA and OA were included in our studies, their effects were comparable to those of PA and LA, respectively. Due to space constraints, the data of SA and OA are not presented in the figures.

(2) While primary macrophages are used in several mechanistic studies, tumor-associated macrophages (TAMs) are not used. Rather, correlative evidence is provided to connect mechanistic studies in macrophage cell lines and primary macrophages to TAMs.

The role of FABP4 in TAMs has been demonstrated in our previous studies using in vivo animal models¹. Therefore, we did not include TAM-specific data in the current study.

(3) CEBPA and FABP4 clearly regulate LA-induced changes in gene expression. However, whether these two key proteins act in parallel or as a pathway is not resolved by presented data.

Multiple lines of evidence in our studies suggest that FABP4 and CEBPA act as a pathway in LA-induced changes: 1) FABP4-negative macrophages exhibit reduced expression of CEBPA in

single cell sequencing data; 2) FABP4 KO macrophages exhibited reduced CEBPA expression; 3) LA-induced CEBPA expression in macrophages was compromised when FABP4 was absent.

(4) It is very interesting that FABP4 regulates both lipid droplet formation and lipolysis, yet is unclear if the regulation of lipolysis is direct or if the accumulation of lipid droplets - likely plus some other signal(s) - induces upregulation of lipolysis genes.

Yes, it is likely that tumor cells induce lipolysis signals. Multiple studies have shown that various tumor types stimulate lipolysis to support their growth and progression²⁻⁴. In this process, lipid-loaded macrophages have emerged as a promising therapeutic target in cancer^{5, 6}. Consistent with findings that lipolysis is essential for tumor-promoting M2 alternative macrophage activation⁷, our data using FABP4 WT and KO macrophages demonstrate that FABP4 plays a critical role in LA-induced lipid accumulation and lipolysis for tumor metastasis.

(5) In several places increased expression of genes coding for enzymes with known functions in lipid biology is conflated with an increase in the lipid biology process the enzymes mediate. Additional evidence would be needed to show these processes are in fact increased in a manner dependent on increased enzyme expression.

We fully agree with the reviewer that increased gene expression does not necessarily equate to increased activity. The key finding of this study is that FABP4 plays a pivotal role in linoleic acid (LA)-mediated lipid accumulation and lipolysis in macrophages that promote tumor metastasis. Numerous lipid metabolism-related genes, including FABP4, CEBPA, GPATs, DGATs, and HSL, are involved in this process. While it was not feasible to verify the activity of all these genes, we confirmed the functional roles of key genes like FABP4 and CEBPA through various functional assays, such as gene silencing, knockout cell lines, lipid droplet formation, and tumor migration assays. Supported by established lipid metabolism pathways, our data provide compelling evidence that FABP4 functions as a crucial lipid messenger, facilitating unsaturated fatty acid-driven lipid accumulation and lipolysis in tumor-associated macrophages (TAMs), thus promoting breast cancer metastasis.

Reviewer #2:

Overall, there is solid evidence for the importance of FABP4 expression in TAMs on metastatic breast cancer as well as lipid accumulation by LA in the ER of macrophages. A stronger rationale for the exclusive contribution of unsaturated fatty acids to the utilization of TAMs in breast cancer and a more detailed description and statistical analysis of data will strengthen the findings and resulting claims.

We greatly appreciated the positive comments from Reviewer #2. In our study, we evaluated the effects of both saturated and unsaturated fatty acids (FA) on lipid metabolism in macrophages. Our results showed that unsaturated FAs exhibited a preference for lipid accumulation in macrophages compared to saturated FAs. Further analysis revealed that unsaturated LA, but not saturated PA, induced FABP4 nuclear translocation and CEBPA activation, driving the TAG synthesis pathway. For in vitro experiments, statistical analyses were performed using a two-tailed, unpaired student t-test, two-way ANOVA followed by Bonferroni's multiple comparison test, with GraphPad Prism 9. For experiments analyzing associations of FABP4, TAMs and other factors in breast cancer patients, the Kruskal-Wallis test was applied to compare differences across levels of categorical predictor variable. Additionally, multiple linear regression models were used to examine the association between the predictor variables and outcomes, with log transformation and Box Cox transformation applied to meet the normality assumptions of the model. It is worth noting that in some experiments, only significant differences were observed in groups treated with

unsaturated fatty acids. Non-significant results from groups treated with saturated fatty acids were not included in the figures.

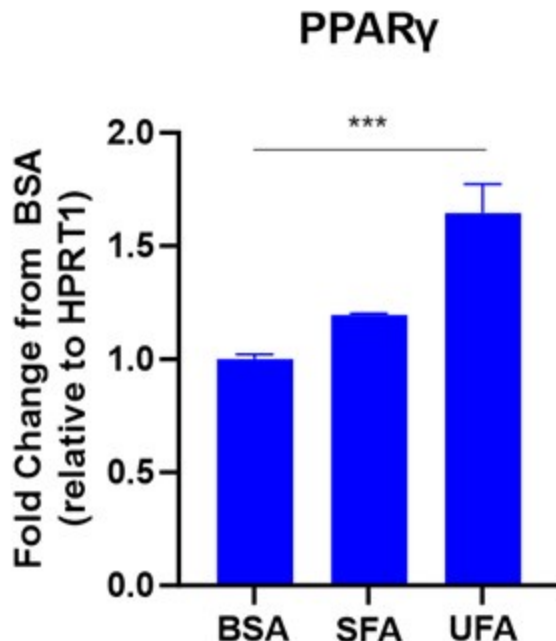
Reviewer #3

(1) While the authors speculate that UFA-activated FABP4 translocates to the nucleus to activate PPARGamma, which is known to induce C/EBPalpha expression, they do not directly test involvement of PPARGamma in this axis.

Yes, LA induced FABP4 nuclear translocation and activation of PPARGamma in macrophages (see Figure below). Since these findings have been reported in multiple other studies 8, 9, we did not include the data in the current manuscript.

Author response image 1.

LA induced PPARG expression in macrophages. Bone-marrow derived macrophages were treated with 400μM saturated FA (SFA), unsaturated FA (UFA) or BSA control for 6 hours. PPARG expression was measured by qPCR (**p<0.001).



(2) While there is clear in vitro evidence that co-cultured murine macrophages genetically deficient in FABP4 (or their conditioned media) do not enhance breast cancer cell motility and invasion, these macrophages are not bonafide TAM - which may have different biology. Use of actual TAM in these experiments would be more compelling. Perhaps more importantly, there is no in vivo data in tumor bearing mice that macrophage-deficiency of FABP4 affects tumor growth or metastasis.

In our previous studies, we have shown that macrophage-deficiency of FABP4 reduced tumor growth and metastasis in vivo in mouse models¹.

(3) Related to this, the authors find FABP4 in the media and propose that macrophage secreted FABP4 is mediating the tumor migration - but don't do antibody neutralizing experiments to directly demonstrate this.

Yes, we have recently published a paper of developing anti-FABP4 antibody for treatment of breast cancer in mouse models¹⁰.

(4) No data is presented that the mechanisms/biology that are elegantly demonstrated in the murine macrophages also occurs in human macrophages - which would be foundational to translating these findings into human breast cancer.

Thanks for the excellent suggestions. Since this manuscript primarily focuses on mechanistic studies using mouse models, we plan to apply these findings in our future human studies.

(5) While the data from the human breast cancer specimens is very intriguing, it is difficult to ascertain how accurate IHC is in determining that the CD163+ cells (TAM) are in fact the same cells expressing FABP4 - which is central premise of these studies. Demonstration that IHC has the resolution to do this would be important. Additionally, the in vitro characterization of FABP4 expression in human macrophages would also add strength to these findings.

The expression of FABP4 in CD163+ TAM observed through IHC is consistent with our previous findings, where we confirmed FABP4 expression in CD163+ TAMs using confocal microscopy. Emerging evidence further supports the pro-tumor role of FABP4 expression in human macrophages across various types of obesity-associated cancers¹¹⁻¹³.

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<https://doi.org/10.7554/eLife.101221.1.sa0>