Fatty Acid-Binding Protein E-FABP Restricts Tumor Growth by Promoting IFN-β Responses in Tumor-Associated Macrophages

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Abstract

Fatty acid-binding proteins (FABP) are known central regulators of both metabolic and inflammatory pathways, but their role in tumor development remains largely unexplored. Here, we report that host expression of epidermal FABP (E-FABP) protects against mammary tumor growth. We find that E-FABP is highly expressed in macrophages, particularly in a specific subset, promoting their antitumor activity. In the tumor stroma, E-FABP-expressing tumor-associated macrophages (TAM) produce high levels of IFN-β through upregulation of lipid droplet formation in response to tumors. E-FABP-mediated IFN-β signaling can further enhance recruitment of tumoricidal effector cells, in particular natural killer cells, to the tumor stroma for antitumor activity. These findings identify E-FABP as a new protective factor to strengthen IFN-β responses against tumor growth.

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Introduction

Fatty acid-binding proteins (FABP) constitute a family of intracellular lipid chaperones coordinating the distribution and function of lipids inside cells (1, 2). It has been well documented that FABPs play central roles in regulating metabolic and inflammatory pathways in various metabolic and autoimmune diseases (3–7). Given the dysregulated metabolic and inflammatory pathways during cancer development, FABPs have been suggested to participate in cancer initiation and progression. However, the exact mechanisms and functions of FABPs in these processes remain largely unknown. In our research focusing on epidermal FABP (E-FABP) functions, we have demonstrated that this protein is highly expressed in immune cells, especially in antigen-presenting cells (APC) and T cells, and regulates both innate and adaptive immune responses (6, 8). Thus, we propose that E-FABP may link to tumor development through shaping host immune surveillance effects.

There is ample evidence indicating that IFNs are critical in mediating immune surveillance to eradicate transformed cells through their effect on host hematopoietic cells (9, 10). Recent studies demonstrate that tumors can drive the production of IFN-β by host APCs to induce spontaneous adaptive T-cell responses, further supporting the essential role of type I IFNs in antitumor immunity (11, 12). However, these seminal studies raise a number of important questions: (i) What is the specific population in the tumor stroma that can produce IFN-β in response to tumors? (ii) How do the IFN-β-producing cells sense and interact with tumor cells? (iii) Which molecule(s) or signaling pathway(s) is (are) critical in regulating IFN-β production? (iv) How does IFN-β signaling lead to enhanced antitumor immunity? It is clear that tumor associated macrophages (TAM) are the most abundant myeloid cells in tumors that exhibit phenotypic and functional heterogeneity (13–15). TAMs are classically divided into Th1 cytokine-induced M1 macrophages and Th2 cytokine-induced M2 macrophages. Although M1 macrophages have been shown to produce abundant levels of proinflammatory cytokines, including type I IFNs, to perform antitumor activities (14, 16), it remains largely unknown which energetic provider is essential to support their antitumor functions.

Because there is high expression of E-FABP in macrophages and E-FABP, as a lipid chaperon, plays a critical role in regulating immune cell functions, we set out to assess whether host expression of E-FABP impacts tumor growth by shaping the function of the immune surveillance process in the present study. Specifically, we determined whether E-FABP displays a unique expression pattern in different subsets of macrophages and how E-FABP regulates specific macrophage antitumor function by focusing on IFN-β production and signaling.

Materials and Methods

Mice and human samples

E-FABP-deficient (E-FABP−/−) and wild-type (WT) mice (C57BL/6 background) were bred and maintained in the...
animal facility of the Hormel Institute (Austin, MN) in accordance with approved protocols from the Institutional Animal Care and Use Committee (University of Minnesota, Austin, MN). Mouse E0771 cells were from CH3 BioSystems; MC38 and RMA cells were gifts from Jun Yan (University of Louisville, Louisville, KY). All cells were cultured less than 6 months for experiments. Cells were not further authenticated. Invasive breast cancer tissue microarray slides were purchased from U.S. Biomax Inc. Human serum samples were collected from patients with benign breast diseases or invasive breast cancers. All patients provided informed consent under an Institutional Review Board-approved protocol.

**Syngeneic mouse models**

Different dosages of E0771 cells were orthotopically implanted into the mammary fat pad of 6- to 8-week-old WT and E-FABP<sup>−/−</sup> mice. Tumors were measured at 3-day intervals with calipers and the volume was calculated by the formula \(0.4 \times \text{large diameter} \times \text{small diameter}^2\). E0771 cells were also intravenously injected into E-FABP<sup>−/−</sup> and WT mice to observe tumor metastasis in lungs. For natural killer (NK) cell- or CD4<sup>+</sup>T-cell depletion assay, mice were intraperitoneally injected with anti-NK1.1 or anti-CD4 neutralizing monoclonal antibody, respectively. After confirmation of the depletion of NK cells or CD4<sup>+</sup>T cells, mice were implanted with E0771 cells as described above. To measure immune cell infiltration and functions in tumor stroma, single cells from solid tumors were prepared by digesting dissected tumors in an enzyme mixture (0.5 mg/mL collagenase A, 0.2 mg/mL type V hyaluronidase, and 0.02 mg/mL DNase I in RPMI-1640) at 37°C for 45 minutes. The separated cells were washed for further analyses.

**Flow cytometric analysis and cell sorting**

Surface and intracellular staining were performed as previously described (8). Single immune cell populations in spleens or tumors were separated with a BD FACSaria II Cell Sorter. Flow cytometric analyses were performed with FlowJo (Tree Star). The detailed antibody clone information is shown in the Supplementary Materials and Methods.

**Gene expression microarray**

A total of \(1 \times 10^6\) granulocyte macrophage colony-stimulating factor-induced bone marrow-derived macrophages (GM-BMM) from WT and E-FABP<sup>−/−</sup> mice were cocultured with \(2 \times 10^6\) E0771 cells in a Transwell plate for 24 hours. Total RNA was extracted from tumor-stimulated GM-BMMs using RNeasy Mini Kit (Qiagen) and subjected to mouse mRNA Gene expression by Affymetrix microarray. Microarray analysis was performed using R/Bioconductor packages and signaling pathway was generated with Ingenuity Pathways Analysis (IPA;...
Ingenuity Systems). The expression profiling data were submitted to the Gene Expression Omnibus (GEO; GSE54073).

**Quantitative real-time PCR**
RNA was extracted from cultured or purified primary cells using RNeasy Mini Kit (Qiagen). cDNA synthesis was performed with QuantiTect Reverse Transcription Kit (Qiagen). Quantitative PCR was performed with SYBR Green PCR Master Mix using ABI 7500 Real-Time PCR Systems (Applied Biosystems). Detailed primer sequences are shown in the Supplementary Information.

**Western blotting**
To quantify the protein levels of E-FABP, A-FABP, phosphorylation, and total STAT1 and STAT2, macrophages with designated treatments were lysed in buffers with protease and phosphorylation inhibitors. Protein concentration was determined by BCA assay (Thermo Scientific). β-actin was used as a loading control. Mouse E-FABP, A-FABP, and β-actin antibodies were from R&D Systems. Mouse STAT1, STAT2, and their phosphorylation antibodies were from Cell Signaling Technology.

**Confocal analysis**
GM-BMMs cultured on poly-D-lysine–coated coverslips (NeuVitro) in a 24-well plate were treated with saturated FAs (stearic acid) or unsaturated FAs (oleic/linoleic acid) for 24 hours. After fixation and permeabilization, the cells were stained with BODIPY 993/590 (Invitrogen) and/or viperin (Millipore). Nuclei were stained with 0.2 μmol/L 4′, 6-diamidino-2-phenylindole (DAPI; Invitrogen). For lipid droplet inhibition assay, GM-BMMs were treated with designated Triacin C plus unsaturated FAs for 24 hours before analysis. For measurement of E-FABP expression, macrophages or breast cancer tissue microarray slides were stained with E-FABP–specific antibody (R&D Systems). Confocal analysis was performed with Nikon Eclipse TE2000 confocal microscopy.

**ELISA**
GM-BMMs (0.5 × 10⁶) from WT and E-FABP⁻/⁻ mice were cocultured with 1 × 10⁶ E0771 tumor lysates (obtained after freezing in liquid nitrogen and thawing at 37°C for 3–4 cycles) for 3 hours. The supernatants were collected for measurement of IFN-β with ELISA Kit (Biolegend). For analysis of IFN-β production in E-FABP knockdown macrophages, macrophages were transfected with On-target plus E-FABP siRNA or scramble oligos (Thermo Dharmacon) using Lipofectamine RNAiMAX (Life Technologies) and stimulated with lipopolysaccharide (LPS; 100 ng/mL) before supernatant collection.

**Tumor killing assays**
Lympocytes collected from draining lymph nodes (dLN) of E0771-tumor bearing mice were used as effector cells. Target cells (E0771) were labeled with 2 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE). Effector and target cells were cocultured for 22 hours in a 96-well v-bottom plate in the presence or absence of blocking antibodies to TRAIL, CD4, CD8, and NK cells, respectively. All cells were stained with 7-aminoactinomycin D (7-AAD) and analyzed by flow cytometry. Tumor-specific killing was calculated as the difference between total percentage of killed (7-AAD⁻) and percentage of spontaneous apoptotic E0771 cells in all CFSE⁺ tumor cells.

**Statistical analysis**
For analysis of microarray data, Affy and Limma packages were used to perform the data prepping steps and implement linear models for differential expression. A false discovery rate corrected P value was used to filter out the differentially expressed probes. Unpaired, two-tailed Student t test was performed for the comparison of results from other different treatments. P < 0.05 is considered statistically significant.

**Results**
**Host expression of E-FABP suppresses tumor growth**
To determine the contribution of host expression of E-FABP to tumor growth, E0771 tumor cells, originally from a C57BL/6 mouse mammary adenocarcinoma (17), were orthotopically injected into the mammary fat pad of WT and E-FABP⁻/⁻ mice and their WT littermates. Tumor growth was measured in a 3-week period after tumor implantation (Fig. 1A). Mammary tumors in E-FABP⁻/⁻ mice grew 3-fold faster as compared with those in WT mice (Fig. 1B). Final tumor weights in E-FABP⁻/⁻ mice were also 3-fold heavier than tumors that formed in WT mice (Fig. 1C). Furthermore, E-FABP deficiency markedly increased E0771 tumor lung metastasis as demonstrated by metastatic spots (P = 0.011; Fig. 1D) and tumor size (Fig. 1E). Experiments with tail vein injection of E0771 cells also demonstrated increased lung tumor burden in the absence of E-FABP (Fig. 1F and G). Of note, when we titrated the dosage of injected tumor cells in mice, we found that the more E0771 cells we injected, the less pronounced was the difference in tumor growth between WT and E-FABP⁻/⁻ mice (Supplementary Fig. S1A), suggesting that host expression of E-FABP does not provide unlimited protection to tumor challenges. To further confirm the protective role of E-FABP, we evaluated tumor burden with other C57BL/6-derived murine tumor cells, that is, the lymphoma cell line, RMA, and the colon cancer cell line, MC38, in E-FABP⁻/⁻ and WT mice. Similar to E0771 cells, these additional cell lines exhibited increased tumor burden.
Figure 3. E-FABP is highly expressed in specific subsets of macrophages. A, identification of CD11b+ F4/80+ macrophages from mouse spleen. B, distinct subsets of splenic macrophages by Ly6C and MHC-II staining. Analyses of CD11c expression by flow histogram (C) and mean fluorescent intensity (MFI; D), and E-FABP expression by real-time PCR (E) in distinct macrophage subsets. F, flow cytometric analysis of dynamic changes of distinct subsets of TAMs in tumor stroma at indicated time points after E0771 cell implantation. Analysis of CD11c expression by MFI (G) and E-FABP expression by real-time PCR (H) in distinct subsets of TAMs on day 7 post E0771 cell implantation.
when E-FABP was absent in mice (Supplementary Fig. S1B and S1C). These results clearly show that host expression of E-FABP is critical in suppressing tumor growth.

E-FABP is highly expressed in macrophages

To understand how E-FABP contributes to tumor protection, we first examined the expression profile of E-FABP in naïve mice. F4/80+ macrophages expressed the highest levels of E-FABP among splenic leukocyte subsets, 9-fold higher than adipocytes (Fig. 2A). Immunostaining of mammary fat tissue (Fig. 2B) and purified populations from peripheral blood (Fig. 2C) showed that E-FABP was also highly expressed in F4/80+ cells. Using bone marrow-derived macrophages, we further confirmed that E-FABP was specifically located in the cytoplasm of WT macrophages, but not in E-FABP−/− cells (Fig. 2D and E). Next, we compared immune cell phenotypes of naïve and E0771 tumor-bearing mice with a focus on TAMs. Compared with spleens and dLNs in naïve mice, tumor-bearing mice exhibited both enlarged spleens and dLNs with significantly higher numbers of total cells (Supplementary Fig. S2A and S2B). Further analysis showed that the enlarged spleens and dLNs were associated with increased CD11b+ F4/80+ macrophages, but not with CD4+, CD8+, NK1.1+, and CD11c+ populations (Fig. 2F and G). Consistently, we found a large quantity of myeloid-derived macrophages (CD11b+ F4/80+) infiltrated in tumors from WT and E-FABP−/− mice (Fig. 2H and I and Supplementary Fig. S2C). As TAMs play critical roles during cancer development (13, 18–20), these results suggest that E-FABP expression in macrophages may protect against tumor growth by promoting their antitumor mechanisms.

Identification of E-FABP expression in specific subsets of macrophages

It is well known that TAMs consist of distinct subpopulations with specialized functions in tumors (21–23). We speculated that E-FABP might exhibit unique expression patterns in different subsets of TAMs. First, we used anti-Ly6C and anti-MHCII antibodies to identify distinct subsets of splenic macrophages (CD11b+ F4/80+) from naïve mice (Fig. 3A and B), and found that each subset exhibited different levels of E-FABP expression (Fig. 3E). Next, we compared immune cell phenotypes of naïve and E0771 tumor-bearing mice with a focus on TAMs. Compared with spleens and dLNs in naïve mice, tumor-bearing mice exhibited both enlarged spleens and dLNs with significantly higher numbers of total cells (Supplementary Fig. S2A and S2B). Further analysis showed that the enlarged spleens and dLNs were associated with increased CD11b+ F4/80+ macrophages, but not with CD4+, CD8+, NK1.1+, and CD11c+ populations (Fig. 2F and G). Consistently, we found a large quantity of myeloid-derived macrophages (CD11b+ F4/80+) infiltrated in tumors from WT and E-FABP−/− mice (Fig. 2H and I and Supplementary Fig. S2C). As TAMs play critical roles during cancer development (13, 18–20), these results suggest that E-FABP expression in macrophages may protect against tumor growth by promoting their antitumor mechanisms.

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TAM subsets exhibited dynamic changes during tumor development (Fig. 3F). Among the distinct subsets, we found that the Q2 subset, peaking on day 7 after tumor implantation (Supplementary Fig. S3A), expressed the highest levels of CD11c (Fig. 3G) and E-FABP (Fig. 3H). Although the Q2 subset declined progressively thereafter, E-FABP expression in this subset remained at higher levels relative to the same subset in the spleen during tumor development (Supplementary Fig. S3B). As the MHCII$^{+}$CD11c$^{+}$ macrophages display a more M1-like phenotype while the MHCII$^{+}$CD11c$^{-}$ subset is more M2 oriented in tumors (14, 23), our data demonstrate a unique expression pattern of E-FABP in M1-like macrophages, suggesting distinct functions of this subset through expression of E-FABP.

Promotion of IFN-β responses in macrophages by E-FABP

As GM-BMMs expressed high levels of CD11b, F4/80, MHCII, CD11c, and E-FABP expression (Supplementary Fig. S4A–S4D), exhibiting a similar phenotype to E-FABP–expressing TAMs as described above, we thus used GM-BMMs from WT and E-FABP–expressing macrophages to dissect how E-FABP expression in specific TAMs contributes to their antitumor activity. We first performed Affymetrix microarray experiments to analyze the E0771 tumor-stimulated gene expression profiles with these cells. The selected differentially expressed genes with P < 0.05 between WT and E-FABP$^{−/−}$ macrophages are shown in the Supplementary Table S1. A heatmap of 33 most differentially expressed genes in response to E0771 stimulation between WT and E-FABP$^{−/−}$ GM-BMMs is shown in Fig. 4A. Very intriguingly, IPA pathway analysis suggested that E-FABP expression in macrophages primarily regulates inflammatory responses, especially IFN-β responses (Supplementary Fig. S4E). We further confirmed the microarray results with real-time PCR. E-FABP–expressing macrophages exhibited significant upregulation of IFN-β–response-related genes, such as viperin, IRF7, IFN-β, CXCL10, CXCL11, Lipg1, and other M1-related molecules, such as TNFSF10 (24) and Ms4a4c (25), as compared with E-FABP$^{−/−}$ macrophages (Fig. 4B and Supplementary Fig. S5F). Moreover, the levels of secreted IFN-β in supernatants of E-FABP$^{−/−}$ TAMs were significantly lower than that in WT macrophages with stimulation of tumor lysates (Fig. 4C). Knockdown of E-FABP with E-FABP siRNA also inhibited IFN-β production in macrophages (Fig. 4D and E). Altogether, these results indicate that E-FABP is essential to promote IFN-β responses in macrophages.

E-FABP regulates IFN-β responses through controlling lipid droplet formation

Because lipid droplets function as platforms to bind viperin, which controls type I IFN induction (26, 27), we speculate that E-FABP as a lipid transporter may control lipid droplet formation to regulate IFN-β responses. As GM-BMMs predominantly expressed E-FABP (Supplementary Fig. S5A and S5B), we used these cells to dissect the effect of E-FABP–mediated lipid droplets for IFN-β production. Compared with saturated stearic acid, unsaturated oleic/linoleic acids were more effective in enhancing E-FABP expression and lipid droplet formation in WT GM-BMMs. More importantly, FA-enhanced lipid droplet formation was markedly decreased in the absence of E-FABP in macrophages (Fig. 5A and B). In line with this observation, the production of viperin and IFN-β was significantly decreased in E-FABP$^{−/−}$ macrophages as compared with WT macrophages (Fig. 5C and D). To determine the essential role of lipid droplets for IFN-β production, we inhibited lipid droplet formation in macrophages with Triacsin C (28), a potent lipid droplet inhibitor (Supplementary Fig. S5C), and confirmed that lipid droplet inhibition indeed impaired IFN-β production in macrophages (Fig. 5E). As oleic acid and linoleic acid are the two most common unsaturated FAs derived from tumor membranes (29), we cocultured macrophages with E0771 tumor cells and demonstrated that E-FABP enhanced lipid droplet formation and viperin production in tumor-stimulated macrophages (Fig. 5F and G). To measure whether E-FABP regulates IFN-β signaling, we found that E-FABP$^{−/−}$ macrophages exhibited reduced phosphorylation of STAT1 and STAT2 as compared with WT cells in response to IFN-β stimulation (Fig. 5H and I). Consistently, when the Q2 subset of TAMs was separated from early-stage E0771 tumors, TAMs from WT mice exhibited markedly enhanced levels of E-FABP, viperin, IRF7, IFN-β, CXCL10, and CXCL11 as compared with the same population from E-FABP$^{−/−}$ mice (Fig. 5J–O), further verifying the attenuated IFN-β responses by E-FABP deficiency in TAMs. Thus, E-FABP can regulate IFN-β responses through controlling lipid droplet formation in specific TAMs.

IFN-β signaling enhances recruitment of tumoricidal effector cells

It has been clear that inflammatory chemokines, such as CXCL10 and CXCL11, can recruit effector T cells and NK cells to inflamed tissues (30, 31), which produce a large amount of IFN-γ for immune surveillance. As IFN-β–producing TAMs highly expressed CXCL10 and CXCL11 in WT mice, we speculated that more effector T cells and NK cells accumulated in these mice to promote their antitumor activity. Indeed, we observed a significantly higher percentage of CD4$^{+}$ T cells, CD8$^{+}$ T cells, and NK cells in tumors from WT mice than in tumors from E-FABP$^{−/−}$ mice in the first week posttumor implantation (Fig. 6A–C). Notably, the difference disappeared in the late stage of tumor growth (3 weeks post E0771 cell

Figure 5. E-FABP promotes IFN-β responses by enhancing lipid droplet formation in macrophages. GM-BMMs were cultured with or without saturated FAs (stearic acid, 100 μmol/L) or unsaturated FAs (oleic/linoleic acid, 25 μmol/L) for 24 hours. E-FABP expression (A), lipid droplet formation (B), viperin (C), and IFN-β (D) expression in these cells were measured by real-time PCR or confocal microscopy. E, analysis of IFN-β levels in supernatants of unsaturated FA-treated macrophages in the absence or presence of indicated lipid droplet inhibitor (Triacsin C) by ELISA. Analysis of lipid droplet formation (F) and viperin expression (G) in GM-BMMs after coculture with or without E0771 tumor cells in a Transwell for 18 hours by confocal microscopy. Analysis of phosphorylation of STAT1 (H), STAT2 (I), and their total proteins in GM-BMMs after stimulation with 100 U IFN-β for the indicated time periods. Expression of E-FABP (J), viperin (K), IRF7 (L), IFN-β (M), CXCL10 (N), CXCL11 (O) in the Q2 subset of TAMs (separated from tumors on day 7 post E0771 cell implantation) was analyzed by real-time PCR. Data represent mean ± SD (*, P < 0.05; **, P < 0.01).
implantation; Fig. 2I), implying an early effect of E-FABP protection. In addition, E-FABP deficiency had no significant impact on IFN-γ production from these infiltrating cells (Supplementary Fig. S6A–S6C). To determine whether these effector cells were responsible for tumor cytotoxicity, we cocultured them with CSFE-labeled tumor cells and demonstrated that effector cells from WT dLNs exhibited higher tumor killing activity than those from E-FABP−/− dLNs (Fig. 6D). Assays with blocking antibodies specific to CD4, CD8, NK, or TRAIL (TNFSF10) suggested that NK cells were the main effector cells in mediating tumor killing in vitro (Fig. 6E). To confirm this observation, we depleted NK cells in WT and E-FABP−/− mice (Fig. 6F and Supplementary Fig. S6D) and demonstrated that the tumor-suppressive effect of E-FABP in WT mice

Figure 6. E-FABP enhances recruitment of tumoricidal effector cells. Analysis of infiltrated CD4+ T cell (A), CD8+ T cells (B), and NK cells (C) in tumors on day 7 post E0771 cell implantation in mice by flow staining. The percentage of each population was shown in the right panel. D, target cells (E0771) were labeled with CFSE and cocultured with effector cells (collected from dLNs of E0771-tumor bearing mice) at the indicated ratio. The percentage of specific tumor killing was analyzed by a flow cytometer. E, tumor-specific killing assays as described in D (effect/target ratio:100) were performed in the absence or presence of respective blocking antibody to CD4, CD8, NK1.1, and TRAIL. F, percentage of NK cells in spleen, peripheral blood, and lymph nodes in mice with or without NK-cell depletion. G, measurement of tumor size 2 weeks after E0771 cell implantation in WT and E-FABP−/− mice with or without NK-cell depletion.
diminished in the absence of NK cells (Fig. 6G). In contrast, depletion of CD4^{+} T cells did not affect tumor growth in both WT and E-FABP^{−/−} mice (Supplementary Fig. S7). Thus, E-FABP−regulated IFN-β signaling in specific TAMs can recruit effector cells, particularly NK cells, for tumor killing.

**E-FABP expression in TAMs in human breast cancers**

Because of the tumor protective role of E-FABP in mouse models, we next examined whether E-FABP was downregulated in human breast cancers. We analyzed E-FABP expression in normal and malignant breast tissues using publicly accessible microarray data. We found that E-FABP was significantly decreased in various types of breast cancers, including invasive ductal carcinoma, invasive lobular carcinoma, and mucinous carcinoma, as compared with normal tissues (Fig. 7A). Interestingly, the statistical significance positively correlated with the sample size (Supplementary Table S2), suggesting a real effect of E-FABP reduction in breast cancers. By analyzing E-FABP expression in breast stroma tissues with a GEO dataset GSE9014 (20), we found that E-FABP was markedly downregulated in stroma of invasive human breast tumors as compared with normal stroma (Fig. 7B and Supplementary Table S3), further corroborating the importance of E-FABP expression in tumor stroma for breast cancer protection. To dissect the location of E-FABP expression in tumor stroma, we used confocal microscopy staining to analyze the expression pattern of E-FABP and TAMs in a tissue microarray containing samples from women with different stages of disease. We found that E-FABP was highly expressed in TAMs of women with early-stage disease, decreasing with disease progression (Fig. 7C and D), which was similar to what we observed in mouse models. In addition, we measured circulating levels

![Figure 7. E-FABP expression in TAMs of human breast cancer. A, analysis of E-FABP expression in normal breast tissues and various types of malignant breast tissues from the publicly accessible microarray databases (https://www.oncomine.org/). B, E-FABP expression in the stroma of normal and malignant breast tissues by analyzing GEO dataset GSE9014. C, costaining of E-FABP expression (red) and TAMs (green) in different stages of invasive breast cancer tissues by confocal microscopy. H&E staining of the same section was shown on the left panel. D, numbers of E-FABP^{+} TAMs per high-power fields (magnification, ×400) were shown as mean ± SD. *, P < 0.05 as compared with stage III invasive breast cancers.](https://www.aacrjournals.org/cancerres/article-pdf/74/11/2995/4388904/can-13-2689.pdf)
of E-FABP in patients with benign breast diseases or patients with breast cancer and found no differences between them (Supplementary Fig. S8A and S8B). Addition of soluble E-FABP directly to the culture media impacted neither the migration of breast cancer MDA-MB-231 cells nor their colony formation (Supplementary Fig. S8C and S8D). These results suggest that circulating E-FABP is not associated with breast cancer development. Altogether, our findings support the idea that E-FABP exerts its protective role through regulation of TAM functions.

Discussion

Compelling evidence from mouse and human studies indicates the importance of immune surveillance of tumors. Thus, how to strengthen host immunity to protect against tumor development remains an area of intense research. It is clear that optimal immune surveillance function depends on appropriate energetic support and homeostasis (34). We and others have observed that E-FABP is expressed highly in macrophages, less in adipocytes, and is barely detectable in noninvasive breast cell lines (35), indicating that E-FABP plays an important role in regulating macrophage functions. Although recent studies suggest that E-FABP ectopic expression in tumor cells can promote their invasive phenotype (36, 37), our data clearly demonstrate that E-FABP as a cytoplasmic energy carrier enhances the effects of macrophage immune surveillance by promoting lipid droplet formation and IFN-β responses in response to tumors. Thus, E-FABP expression in macrophages represents a new mechanism to protect against tumor growth.

To dissect how E-FABP enhances macrophage antitumor activity, we investigated E-FABP expression in different subsets of macrophages and identified that E-FABP is highly expressed in a specific subset, which exhibits the CD11b−F4/80+Ly6c−CD11c+MHCIi phenotype (Fig. 3). More intriguingly, this subset reached peak levels in the tumor stroma in the first week of posttumor implantation and declined thereafter, implying that E-FABP–mediated protection is initiated in the early stage of tumor development. Although not influencing TAM infiltration, E-FABP expression in specific TAMs promotes their production of IFN-β. Consistent with previous studies identifying CD11c+ APCs as the source of IFN-β production in tumors (12, 38), our data further define the phenotype of these IFN-β–producing cells and identify the importance of E-FABP in mediating IFN-β responses in this specific subset.

The continuous death of malignant cells during the tumorigenic process results in uptake of macrophages as well as a range of immune responses within the tumor (39). Although much attention has been focused on tumor-released proteins and nucleic acids in triggering host immune responses (40, 41), it is largely unknown how macrophages deal with the large pieces of lipid debris produced from dying tumor cells. In exploring E-FABP as a new sensing factor mediating macrophage–tumor interactions, our data suggest that specific macrophages can use tumor-derived lipids through upregulation of E-FABP to support their antitumor function. Mechanistically, E-FABP–mediated lipid transportation enhances energy storage and lipid droplet formation in macrophages. Lipid droplets not only provide lipids for energy metabolism, but also regulate intracellular immune responses through binding specific signaling proteins (42). It has been shown that viperin, an IFN-stimulated protein, is anchored with its N-terminal α-helix domain to lipid droplets to mediate IFN-β production (43, 44). Our data provide experimental evidence that macrophage–tumor interaction promotes E-FABP–mediated lipid droplet formation and increases viperin and IFN-β production in macrophages. In contrast, inhibition of lipid droplet formation significantly impairs IFN-β production (Fig. 5). Thus, lipid droplets play an essential role for IFN-β production in macrophages, which contributes to our increasing understanding of lipid droplet functions (45).

IFN responses usually consist of two phases: (i) the production of IFNs by innate cells in response to infection, autoimmunity, or tumor stimulation, and (ii) the binding of secreted IFNs to IFN receptor to activate JAK-STAT signaling and induce IFN-stimulated genes (ISG; ref. 26). Our data demonstrate that E-FABP affects not only the production of IFN-β in response to tumors, but also the phosphorylation of STAT1 and STAT2, and the expression of ISGs, such as CXCL10, CXCL11, CCL12, LIPG1, and TNFSF10 (Figs. 4 and 5). Of note, E-FABP deficiency has no impact on the expression of IFN-β receptor expression (Supplementary Fig. S4F). To explore how E-FABP–regulated IFN-β responses enhance host antitumor immunity, we found that increased numbers of effector cells, including T cells and NK cells, are recruited into the tumor milieu of WT mice. We further identified that NK cells are the major effector cells in mediating tumor immunosurveillance effect of E-FABP (Fig. 6), which is consistent with previous studies showing that NK cells are the main IFN targets for antitumor responses (46, 47). Although E-FABP has been shown to promote Th17-cell differentiation in the model of experimental autoimmune encephalomyelitis (8), we did not observe any significant interleukin-17–producing CD4+ T cells in tumor stroma of the tested tumor models (data not shown). Instead, IFN-γ is largely produced by infiltrated T cells and NK cells, but E-FABP deficiency does not significantly affect IFN-γ production (Supplementary Fig. S6). Thus, E-FABP promotes IFN-β production and signaling in specific macrophages, which further increases the recruitment of effector cells, especially NK cells, for tumor killing, thereby leading to reduced tumor growth. As E-FABP deficiency has no impact on the production of prometastatic molecules (e.g., matrix metalloproteinase, VEGF) in macrophages as indicated from our microarray analyses, it is possible that, compared with E-FABP−/− mice, the reduced tumor lung metastasis in E-FBBP sufficient mice is due to the increased tumor killing in these mice.

Because E-FABP expression strengthens host antitumor immune surveillance, it is logical to speculate that established tumors may exhibit decreased expression of E-FABP. Indeed, E-FABP levels in different types of human breast cancer tissues are significantly lower as compared with normal breast tissues. As our mouse studies indicate that E-FABP is highly expressed...
in macrophages in tumor stroma, further analysis with human breast stroma tissues demonstrated that E-FABP expression is markedly reduced in the stroma of invasive breast cancers. In addition, we confirmed that E-FABP is mainly expressed in TAMs in human breast cancer tissues and the numbers of E-FABP–expressing TAMs are significantly reduced in advanced stages of invasive breast cancers (Fig. 7). Although macrophages are generally considered to contribute to tumor progression through enhancing angiogenesis, migration, and invasion (15, 48), our research points out that a specific subset of TAMs, which highly expresses E-FABP, performs strong antitumor activity.

In summary, we find that E-FABP is highly expressed in a specific subset of macrophages, which produce large amounts of IFN-β triggered by tumors. Mechanistically, E-FABP promotes IFN-β responses through the control of lipid droplet formation during macrophage–tumor interactions, which further enhance recruitment of tumoricidal effector cells for tumor killing. Thus, E-FABP represents a new protective factor in the control of cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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