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IgA Fc-folate conjugate activates and recruits neutrophils to directly target triple negative breast cancer cells

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Abstract

Purpose—According to the American Cancer Society, 1 in 8 women in the U.S. will develop breast cancer, with triple negative breast cancer (TNBC) comprising 15-20% of all breast cancer cases. TNBC is an aggressive subtype due to its high metastatic potential and lack of targeted therapy. Recently, folate receptor alpha (FRA) is found to be expressed on 80% of TNBC with high expression correlating with poor prognosis. In this study, we examined whether binding IgA Fc-folate molecules to FRA receptors on TNBC cells can elicit and induce neutrophils (PMNs), by binding their FcαR1 receptors, to destroy TNBC cells.

Methods—FRA was analyzed on TNBC cells and binding assays were performed using ³H-folate. Fc-folate was synthesized by linking Fc-fragments of IgA via amine groups to folate. Binding specificity and antibody dependent cellular cytotoxicity (ADCC) potential of Fc-folate to FcαR1 was confirmed by measuring PMN adhesion and myeloperoxidase (MPO) release in a cell-

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Conflict of Interest

“The authors declare no conflict of interest.”

Ethical approval

All procedures performed in studies involving human participants were performed in accordance with the Declaration of Helsinki and have been approved following Expedited Review (IRB #123016MP4E) by the Chairperson for the Wayne State University Institutional Review Board (MP4).

based ELISA. Fc-folate binding to FRA-expressing TNBC cells inducing PMNs to destroy these cells was determined using ^{51}Cr -release and calcein-labeling assays.

Results—Our results demonstrate expression of FRA on TNBC cells at levels consistent with folate binding. Fc-folate binds with high affinity to FRA compared to whole IgA-folate and induces MPO release from PMN when bound to Fc α R1. Fc-folate inhibited binding of ^3H -folate to TNBC cells and induced significant cell lysis of TNBC cells when incubated in the presence of PMNs.

Conclusion—These findings support the hypothesis that an IgA Fc-folate conjugate can destroy TNBC cells by eliciting PMN-mediated ADCC.

Keywords

IgA Fc-folate; folate receptor alpha; Fc α R1; triple-negative breast cancer; antibody-dependent cellular cytotoxicity

INTRODUCTION

According to the American Cancer Society, approximately 1 in 8 women in the U.S. will develop breast cancer at some point in their life [1]. Of these women, 15-20% will be diagnosed with triple negative breast cancer (TNBC), an aggressive subtype with a high mortality rate[2]. TNBC refers to breast tumors that lack receptors for estrogen (ER), progesterone (PR), and epidermal growth factor (HER2) and therefore, are not dependent on them for growth. These tumors do not respond to hormonal therapy (e.g., tamoxifen or aromatase inhibitors) or HER2-targeted therapy (e.g., trastuzumab). Of these women who are diagnosed with TNBC, approximately 23% will not survive five years [3]. Lack of effective targeted therapies force clinicians to rely heavily on chemotherapeutics that nonspecifically target replicating cells, resulting in toxic side effects. The most promising targeted treatment for TNBC is olaparib (PARP inhibitor), approved this year for treating advanced breast cancers with BRCA mutations. However, this drug only targets TNBC patients who present with BRCA1/2 gene mutation (approximately 20%) [4]. Thus, there is a clear need for new drugs targeting TNBC, where an unmet need exists with limited targeted therapy approved.

Folate receptor type alpha (FRA) is over-expressed by a majority of cancers including breast, with limited expression in healthy tissue and organs [5, 6]. In normal tissue, FRA is mainly expressed on the luminal surface of epithelial cells, making it inaccessible via the vasculature, thus eliminating toxic side effects of FRA targeted treatments. Recently, two studies found FRA expression to strongly associate with ER/PR-negative and TNBC (>80%) status as well as poor prognosis with FRA expression associated with metastatic breast cancer and worse overall/ disease-free survival [7, 8]. Thus, FRA is an ideal target for directing therapeutic agents to TNBC. In fact, some folate conjugates, such as folate-fluorescein or folate-IgG have shown significant anti-tumor activity in mice [9, 10]. Anti-FRA IgG antibodies such as MORAB-003 (Farletuzumab) have also been reported to target ovarian cancer and have been tested in patients [11]. However, this drug failed to show efficacy in Phase I/II trials. A recent study shows MORAB-003 decreased tumor growth of

ovarian cells by inducing autophagy but not necessarily apoptosis [12] suggesting inhibition of FRA alone is not enough to kill tumor cells. MORAB-003 was shown to induce ADCC in vitro [13]. IgG antibodies stimulate ADCC through binding of their Fc portion to Fc γ III receptors on leukocytes [14, 15]. However, ADCC response mediated through binding of IgG to Fc γ III *in vivo* is weak due to low Fc γ III levels, the presence of high competing serum IgG, the presence of Fc γ III on non-cytotoxic cells and the presence of inhibitory Fc γ II receptors [14, 16]. Thus, IgG monoclonal antibodies are unable to promote effective anti-tumor responses [17, 18] and, because of their large size, have poor tumor penetration [19]. Fc α R1, on the other hand, is only expressed on cytotoxic cells and in high numbers on PMNs making this receptor a very potent IgA-mediator of ADCC independent of cytokine co-factor(s) [20].

In this study, we demonstrate novel binding of IgA Fc-folate conjugates to FRA-expressing TNBC cells. Upon binding, Fc-folate recruits and activates PMNs to destroy TNBC cells by binding Fc α R1 on the PMN and activating ADCC (Fig. 1). Numerous studies document PMNs as potent effector cells for IgA-mediated cellular cytotoxicity [21-28]. These findings suggest an FRA-targeted therapy can effectively kill TNBC cells and may represent an effective, targeted treatment for TNBC.

MATERIALS AND METHODS

Reagents and cell culture

Human SUM TNBC cell lines were a kind gift from Dr. Stephen Ethier. All other TNBC cell lines (MDA-MB-231, MDA-MB-468, BT549) were purchased from ATCC. iGROV1 cells (shFRA and NS) were a kind gift from Dr. Larry Matherly. Sf9 and Hi-Five insect cells were purchased from Thermo Fisher Scientific (Waltham, MA) and grown in SF900 medium supplemented with 10% FBS and Express Five serum-free medium containing 20 mM glutamine, respectively. TNBC cell lines were authenticated via cytogenetic analysis or used within 6 months of purchase or stored in liquid nitrogen for future use. Cell culture reagents were purchased from Thermo Fisher Scientific. IgA from pooled human serum was purchased from Athens Research and Technology (Athens, GA).

Isolation and labeling of PMN from Whole Blood

Heparin anti-coagulated blood was obtained from healthy volunteers after informed consent in accordance with ethical guidelines of Wayne State University. PMNs were isolated using Ficoll-Paque followed by dextran (1%) density gradient centrifugation as described previously [29] and labeled with 10 μ g/ml BCECF-AM (ThermoFisher Scientific) for 30 minutes at 37°C just before use.

Stable transduction of cells with FRA shRNA plasmids

Transduction reagents were purchased from ThermoFisher Scientific. GIPZ Lentiviral particles containing FRA shRNA #4 vector (*shFRA*) or non-silenced control vector (NS) were obtained from Karmanos Cancer Institute by subscription to Thermo Scientific GIPZ shRNA library. Lentiviral particles containing these vectors were generated by reverse transfection of these constructs with Trans-Lentiviral package mix, into HEK293T cells

using Arrest-In/Express-In transfection reagent. A 1:1 dilution of virus produced was used to infect iGROV1 cells in the presence of polybrene (10 µg/ml) and stable cultures generated by growing in puromycin (1 µg/ml). *FRA* silencing was confirmed by RT-qPCR and folate binding assay.

FRA analysis and folate binding

RNA was extracted using RNeasy Plus Mini Kit (Qiagen). Reverse transcription was performed with 2 µg RNA using High-capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). QPCR was performed using ABsolute qPCR Mix (Thermo Scientific) with the following sense/anti-sense oligonucleotide primers:

<i>FRA</i>	Sense	5'-CAT TGC ACA GAA CAG TGG GTG-3'
	Anti-sense	5'-AAG TGC GCA GTG GGA GCT-3'
<i>GAPDH</i>	Sense	5'-ACA ACT TTG GTA TCG TGG AAG G-3'
	Anti-sense	5'-CAG TAG AGG CAG GCA TGA TGT TC-3'

No RT controls were used to confirm lack of contaminating genomic DNA. *FRA* expression was determined using the following equation: $2^{Ct(\text{reference}) - Ct(\text{target})}$ with GAPDH as the reference gene.

Folate binding assays were performed as described [30] and modified [31]. Briefly, cells were plated at 2.5×10^5 cells/well in 24-well plates overnight in folate-free RPMI and 10% FBS containing ~0.02 µM physiological levels of folate (plating medium). Cells were placed on ice, washed twice with cold low pH buffer (20 mM sodium acetate; pH 3.0) to strip remaining folate from cell membrane and then washed two times in cold PBS (3 mM sodium phosphate, 155 mM NaCl, 1 mM potassium phosphate; pH 7.4) prior to incubation in 61 µCi/ml ^3H -folate (100 nM). After one hour incubation, unbound folate was washed from cells and remaining ^3H -folate eluted using 0.5 ml cold low pH buffer and counted using a Perkin Elmer liquid scintillation analyzer. For each cell line, background cpm values from control wells (containing both ^3H -folate and 1 mM cold folate) were subtracted from wells containing only ^3H -folate and resulting cpm values were used, in some cases, to determine nmoles of ^3H -folate bound where results are plotted as average nmoles of ^3H -folate bound per one million cells (counted in parallel). For determining binding of folate conjugates to FRA, binding assays were performed in the presence of varying concentrations of folate conjugates and the amount of conjugate required to displace 50% of ^3H -folate bound to FRA on cells was determined.

Synthesis of IgA Fc-folates

Fc-fragments were generated from pooled human IgA (~80% IgA-1) using Pierce's Fab Preparation Kit (ThermoFisher Scientific) per their instructions. Briefly, immobilized papain resin (0.125 ml) was equilibrated in micro-spin columns with digestion buffer (PBS) and then incubated with 0.5 ml of whole IgA (1 mg/ml) in digestion buffer for 8 hours with end-over-end rotation at 37 °C. After the desired timepoint, digest was separated from resin by

centrifugation and the Fc fragment was affinity purified from the digest using Human IgA Affinity Matrix columns (Thermo Fisher Scientific) which contain antibody fragments recognizing all human IgA subclasses. The affinity resin (0.125 ml) was equilibrated in micro-spin columns with digestion buffer and then incubated with digest (0.5 ml) for 30 minutes with end-over-end rotation at room temperature. Fc fragments retained on the resin were collected by centrifugation followed by four washes with PBS (0.5 ml) and eluting with 0.1 μ M glycine (pH 3). Fc fragment purity and yield was determined by SDS-PAGE analysis of both wash and elution fractions which should contain Fab and Fc fragments, respectively.

Folate was attached to whole IgA or Fc fragments by coupling folate to amine groups using 1-ethyl-3-[3dimethylaminopropyl] carbodiimide hydrochloride (EDC) to activate folate according to manufacturer (Pierce). N-hydroxysuccinimide (NHS) was used to stabilize the acylisourea folate intermediate. Briefly, folic acid (1 mg/ml) in DMF and activation buffer (0.1M MES, 0.5M NaCl, pH 6.0) at a ratio of 1:1 was activated by adding EDC (10-fold molar excess) and NHS (2.5 molar excess to EDC) and incubating for 15 minutes at room temperature in the dark. After incubation, EDC was quenched by addition of 20 mM BME and IgA or Fc fragments in coupling buffer (100mM sodium phosphate, 150mM NaCl; pH 7.2) were added and allowed to incubate at room temperature for two hours. The reaction was quenched by addition of hydroxylamine (10 mM) and excess activated folate removed using 7kD Zeba desalting spin columns (Thermo Scientific).

Fc-folate binding to Fc α R1 and MPO release

Binding specificity of Fc-folate to Fc α R1 was determined by cell-based ELISA assay. To do this, ELISA plates were triplicate coated overnight with IgA or Fc fragments, their corresponding folate conjugates, or bovine serum albumin (negative control) in PBS at varying doses. After incubation, unattached protein was washed away and freshly isolated and BCECF-labeled PMNs were added at 4×10^5 cells/well in plating medium and allowed to attach. After 30 minutes, wells were PBS washed and remaining PMNs detected by lysing in 2% SDS, transferring to black plates and measuring BCECF fluorescence (ex: 485/20 and em: 528/20) using a BioTek Synergy 2 plate reader. Values represent average absorbance per treatment. Functionality of folate conjugates binding to Fc α R1 was determined by measuring PMN-induced MPO release. Briefly, plates were coated overnight as described above and freshly isolated and unlabeled PMNs were added and incubated for 6 hours. After incubation, supernatants were removed, spun down to remove cellular debris and stored at -80°C until assayed. MPO was measured as previously described [29]. Briefly, 50 μ l of the supernatant fluids containing MPO were incubated in 200 μ l of a potassium phosphate buffer (50 mmol/L) containing the substrate, H_2O_2 (1.5 mol/L) and o-dianisidine dihydrochloride (167 μ g/ml; Sigma Aldrich). Enzyme activity rate was determined spectrophotometrically by measuring the change in absorbance at 460 nm over 10 minutes using a 96-well plate reader. Values represent the average maximum MPO activity (abs/sec).

^{51}Cr -release and calcein-AM cytotoxicity assays

^{51}Cr -release assays were performed as previously described [32]. Briefly, TNBC cells were plated at 1×10^5 cells/well in 96-well plates overnight in plating medium and then labeled

with ^{51}Cr (250 $\mu\text{Ci/ml}$) for 4 hours. Excess ^{51}Cr was washed away with PBS and cells were incubated overnight (12-15 hours) in co-culture with PMNs and in the presence and absence of Fc-folate (3 μM), treated in triplicate, at a target to effector ratio of 1:60 (T:E). Cell lysis was determined by measuring ^{51}Cr released into the supernatant using a PerkinElmer scintillation counter. For each cell line, resulting cpm values in the supernatant from control wells (TNBC cells with no PMNs) were subtracted from all PMN-containing wells. Results are expressed as percent of maximum control lysis where maximum control lysis of each cell line was determined by lysing additional control wells with 2% SDS.

Calcein-AM assays were performed according to manufacturer instructions (ThermoFisher Scientific). Briefly, TNBC cells were plated at 1×10^5 cells/well in 96-well black plates overnight in plating medium and then labeled with calcein-AM dye (1 mg/ml) for 45 minutes. Excess dye was washed away with PBS and cells were incubated for 8 hours in co-culture with PMNs and in the presence and absence of Fc-folate (3 μM), treated in triplicate and at a target to effector ratio of 1:50 (T:E). After 8 hours, wells were washed and remaining adherent cells were lysed with 2% SDS and detected by measuring calcein fluorescence (ex: 485/20 and em: 528/20). Cell viability for each cell line is expressed as percent of control where control represents the individual TNBC cells with no PMN treatment.

Statistical Analyses

Data were analyzed using GraphPad Prism (v.7.0) for Macintosh. All results are expressed as mean $\pm$ SD of three independent experiments unless otherwise stated and statistical analysis performed by one-way or two-way repeated measures analysis of variance (ANOVA) followed by multiple comparison procedure with Student—Newman Keuls method. A value of $P = 0.01$ was considered significant.

RESULTS

TNBC cells express FRA and bind folate

FRA expression was measured by RT-qPCR in various TNBC cells. All TNBC cells tested expressed varying levels of FRA except BT549 cells (Fig. 2a). Folate binding assays utilizing ^3H -labeled folate as described above were performed with the high FRA expressing cells (Fig. 2b). Results demonstrate ^3H -folate binding to TNBC cells at levels consistent with FRA expression. FRA specificity was confirmed in these studies by demonstrating strong binding of ^3H -folate to human ovarian FRA-expressing cancer cells (iGROV1) compared to FRA-silenced iGROV1s.

Binding of IgA- and Fc-folates to FRA

IgA and Fc-folate conjugates were synthesized as described above. Fc fragmentation was confirmed by gel electrophoresis (Fig. 3) showing a band around 30 kd in the elution fraction (Fc fragment) and lack of bands at 25 and 45 kd corresponding to Fab fragments present in high levels in the wash fraction (Fab fragment). The presence of heavy and light chains in the Fc fraction indicates incomplete digestion of IgA. Multiple attempts to achieve complete digestion were attempted without success so the digestion shown above was used

for synthesis of the Fc-folate conjugate. After synthesis, the folate concentration of the conjugates were determined spectrophotometrically at 363 nm and binding to FRA tested in the ^3H -folate binding assay. Resulting cpm values were plotted and amount of conjugate required to displace 50% of ^3H -folate bound to FRA on iGROV1s was determined. Both IgA and Fc-folate conjugates bound to FRA, displacing 100 nM ^3H -folate with IC50 values of 2.8 μM for whole IgA (Fig. 4a). For Fc-folate conjugate, almost complete inhibition was achieved at the lowest concentration tested (0.724 μM). The lowest dose of Fc-folate found to achieve maximum ^3H -folate displacement (3 μM) was used to confirm specificity of ^3H -folate and Fc-folate for FRA (Fig. 4b). Results demonstrate high ^3H -folate binding in FRA-expressing iGROV1 (NTC) with little binding in shFRA-infected iGROV1 confirming specificity of ^3H -folate for FRA. Binding was almost completely inhibited by Fc-folate in FRA-expressing iGROV1 and to the same level as unlabeled folate. In shFRA-infected iGROV1s that bound little ^3H -folate, Fc-folate still significantly inhibited binding. Since whole IgA was present in the Fc-folate conjugate, whole IgA was tested alone and found no interference with ^3H -folate binding confirming folate-binding requirement. Normal human breast epithelial cells (HME6/7) bound very little ^3H -folate consistent with low FRA levels in normal tissue [33, 34]. This binding was inhibited by Fc-folate. These results confirm strong binding and specificity of Fc-folate to FRA.

PMNs bind Fc-folate and stimulate ADCC

Next, to confirm that IgA and Fc-folate conjugates were capable of binding Fc α R1 and stimulating PMNs to induce ADCC, their ability to bind and induce PMNs to release MPO was measured (Fig. 5). After overnight coating of ELISA plates with varying concentrations of either whole IgA, Fc fragment, IgA-folate or Fc-folate, pre-labeled PMNs were added to the plate for 30 minutes, washed to remove non-adherent PMNs and remaining PMNs bound were determined by measuring BCECF fluorescence. In the presence of the lowest dose of Fc-folate (0.75 $\mu\text{g}/\text{ml}$), there was a slight but insignificant increase in PMN adherence (Fig. 5a). However, at a dose of 1.56 $\mu\text{g}/\text{ml}$ and above, both IgA and Fc fragments, as well as their corresponding folate conjugates, induced at least 4-fold significant increase in PMN adherence compared to BSA treated wells with maximum adherence occurring at a dose of 3.125 $\mu\text{g}/\text{ml}$ (Fig. 5a).

Although it is unclear how PMNs mediate ADCC, studies have shown MPO is essential for this process to occur [35]. Therefore, to test for functionality of the folate conjugates, their ability to induce PMNs to release MPO was determined using an ELISA plate coated overnight with both IgA and Fc fragments, as well as their corresponding folate conjugates as described above. Since initial studies showed significant IgA-induced MPO release by PMNs compared to BSA treated wells alone as early as 6 hours after PMN addition, this timepoint was chosen to measure MPO release. Results demonstrate a dose-dependent increase in MPO release by PMNs exposed to both IgA fragment and its folate conjugate with a significant increase compared to BSA alone at a concentration of only 1.56 $\mu\text{g}/\text{ml}$ (Fig. 5b). A dose-dependent increase in MPO release was also triggered by Fc-fragment or its folate conjugate but the response was slightly weaker with a small but significant increase at a concentration of 6.25 $\mu\text{g}/\text{ml}$. The response of the folate conjugates compared to their

fragments alone was not significantly different in either the MPO or adhesion assay, demonstrating that attached folate has no effect on IgA or Fc-fragment binding to FcαR1.

TNBC cells bind Fc-folate and trigger cell lysis

Once binding of the Fc-fragment to FRA expressing iGROV1 was established, ADCC by PMNs due to Fc-folate binding was tested in high FRA-expressing TNBC cells using both the standard chromium (^{51}Cr)-release assay [32] and fluorescent based calcein-AM assay. First, their ability to inhibit binding of ^3H -folate to these cells was determined and results demonstrated a significant inhibitory effect of Fc-folate, at a concentration of 3 μM (Fig. 6a). Using these same cell lines, in addition to iGROV1, ADCC was tested and results demonstrate a significant increase in cell lysis of all TNBC cells tested by the PMNs in the presence of Fc-folate compared to cells cultured with PMNs alone (Fig. 6b-c). The iGROV1s were the most sensitive (100% lysis) which corresponds with their high FRA expression level (Fig. 2a) and lack of lysis in the shFRA-expressing iGROV1s by PMNs confirms FRA requirement. Interestingly, ADCC measured by the calcein-AM assay corresponded more closely with FRA expression in the TNBC cells compared to the ^{51}Cr release assay.

DISCUSSION

Since identification and pioneering studies of FRA [36-40], targeting FRA in ovarian and, recently, breast cancer with various cytotoxics is currently being tested in the clinic. Some of these are in Phase II/III trials for treatment of cancers [41-43]. However, the first Phase III clinical trial incorporating one of these drugs (Vintafolide) was recently halted for lack of efficacy compared to chemotherapy alone [44] and others trials suspended as well [45, 46]. By utilizing FRA only as a target for PMN docking and activation in the present study, we demonstrate that an IgA Fc-folate conjugate can effectively activate PMNs to target and kill FRA-expressing TNBC cells. In a related study, an IgG folate conjugate was shown to elicit and activate NK cells to target FRA-expressing mouse cancer cells [9], further confirming the therapeutic potential of utilizing FRA as a target for immune cell-mediated ADCC. Further studies employing recombinant IgA Fc fragments as a means to increase efficacy of the folate conjugates are currently ongoing and will be interesting to see if altering Fc fragment composition can alter efficacy of these folate conjugates.

The concept of targeting FcαR1 using monoclonal or bispecific IgA antibodies to elicit tumor cell killing by PMNs has been tested successfully *in vitro*, where IgA showed superiority over IgG [21-28] as well as *in vivo* using IgA targeting EGFR antibodies [47, 48]. However, research into the therapeutic use of FcαR1 is hampered by lack of appropriate mouse models (because mice do not express FcαR1 [49]) and lack of established models for production/purification of high levels of IgA [50]. In this study, we demonstrate that an IgA Fc-folate conjugate can bind strongly to FRA receptors on TNBC stimulating PMN mediating cell killing and thus, avoids these obstacles because it utilizes only an Fc portion of IgA so there is no need to make IgA antibodies targeting FRA. We are in the process of developing homozygous BALB/c scid mice with the human FcαR1 gene inserted for *in vivo* testing of recombinant Fc-folate conjugates. Since PMNs are present in breast tumors at

high levels and are cytotoxic towards tumor cells [51], FcαR1 could be an ideal target for other drugs targeting PMN-triggered ADCC in TNBC. Since activation of ADCC through FcαR1 requires IgA complexes, [52] there is no toxicity due to binding of plasma monomeric IgA. The concept of utilizing IgA can be applied to other cancer immunotherapies as well as a means for improving efficacy or for treating autoimmune diseases.

The results of this study strongly support the hypothesis that IgA Fc-folate conjugates can effectively destroy TNBC cells by binding FRA and eliciting PMN-mediated ADCC. Unlike other failed FRA-mediated approaches or those requiring internalization by tumor cells or dependence on an adaptive immune response which is defective in human tumors, Fc-folate conjugates only need to bind FRA to activate PMNs to effectively target TNBC tumors. By doing so, Fc-folates will decrease TNBC patient mortality and will do so with little toxicity because it will only target FRA-expressing tumor cells. Fc-folates can be applied to other aggressive FRA-expressing cancers including ovarian and lung, which also have high mortality rates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fc-folate conjugate triggers neutrophil (PMN) –mediated tumor cell lysis by antibody-dependent cellular-cytotoxicity (ADCC).

Binding of the folate component of the conjugate to its folate receptor (FRA) present on tumor cells allows binding of the Fc fragment to its receptor (FcαR1) on the PMN. This binding will cross-link FcαR1 receptors, triggering ADCC through the release of cytotoxic enzymes resulting in tumor cell death.

Figure 2. FRA expression and ³H-folate binding in TNBC cells.

(A) Cancer cells were grown to confluence in folate-free RPMI and 10% FBS containing ~0.02 mM (physiological level) of folate (plating medium). *FRA* message was measured by RT-qPCR. (B) Cancer cells were plated at 2.5×10^5 cells/well in 24-well plates in plating medium and incubated overnight. Binding experiments were performed on ice in cells stripped of folate by washing with cold low pH buffer (pH 3) twice prior to incubation in 61 μ Ci/ml ³H-folate (100 nM) in PBS for 1 hr. After one hour incubation, cells were washed with PBS to remove unbound folate and remaining ³H-folate eluted using cold low pH buffer and counted using a Perkin Elmer liquid scintillation analyzer. For each cell line, background cpm values from control wells (containing both ³H-folate and 1mM cold folate) were subtracted from wells containing only ³H-folate and resulting cpm values were used to determine nmoles of ³H-folate bound. Results are plotted as average nmoles of ³H-folate bound per one million cells (counted in parallel). Human ovarian cancer cells infected with non-silenced control vector (iGROV1) or sh*FRA* were used to confirm ³H-folate binding specificity. Results represent the mean $\pm$ SD of $n = 3$ independent experiments performed in triplicate.

Figure 3. Confirmation of Fc-fragmentation by gel electrophoresis.

SDS-PAGE (10% Bis-Tris) results of reduced samples from the IgA digest purified using the human IgA affinity matrix. Fc fragmentation was confirmed showing a band around 30 kd in the elution fraction (Fc fragment) and lack of bands at 25 and 45 kd corresponding to Fab fragments which are present at high levels in the wash fraction (Fab fragment). The presence of heavy and light chains in the elution fraction indicates incomplete digestion of IgA.

Figure 4. IgA- and Fc-folate conjugates inhibit binding of ^3H -folate.

(A) iGROV1 cells were plated at 2.5×10^5 cells/well in plating medium. Binding assays were performed as described in Figure 2 but in the presence of increasing amounts of folate conjugates which compete with ^3H -folate for FRA binding. Results represent the mean $\pm$ SD of $n = 3$ independent experiments each performed in duplicate. IC₅₀ values were determined using GraphPad Prism version 6.0. (B) Specificity of ^3H -folate and Fc-folate for FRA was demonstrated using FRA-expressing and shFRA-infected iGROV1s as well as normal human epithelial cells in the same binding assay as described in (A) but using 3 μM Fc-folate conjugate and 1 mg/ml whole IgA. Results represent the mean $\pm$ SD of $n = 3$ independent experiments each performed in duplicate where * $P < 0.01$ compared to ^3H -folate alone.

Figure 5. IgA and Fc-folates bind PMNs and induce ADCC.

(A) ELISA plates were triplicate coated overnight with varying concentrations of IgA, Fc fragment, their corresponding folate conjugates or BSA (negative control) prior to addition of BCECF-AM labeled PMNs at 4×10^5 cells/well in plating medium. After incubation for 30 minutes, unattached PMNs were washed away and remaining cells lysed in 2% SDS, transferred to black plates and fluorescence measured (ex: 485/20 and em: 528/20). Results represent the mean $\pm$ SD of $n = 3$ independent experiments performed in triplicate. (B) PMN-induced MPO release was measured in the clarified supernatant collected 6 hours after plating of unlabeled PMNs in the ELISA experiment described in Part A. Results represent the mean $\pm$ SD of $n = 3$ independent experiments performed in triplicate where $*P < 0.01$ compared to samples containing PMN alone.

Figure 6. TNBC cells bind Fc-folate and trigger cell lysis.

(A) Binding assays were performed in high FRA-expressing TNBC cells as described in Figure 2. Results represent the mean $\pm$ SD of $n = 3$ independent experiments performed in triplicate where * $P < 0.01$ compared to ^3H -folate alone. (B) TNBC cells were plated at 1×10^5 cells/well in 96-well plates overnight in low folate RPMI containing 10% and then labeled with ^{51}Cr (250 $\mu\text{Ci}/\text{ml}$) for 4 hours. Excess ^{51}Cr was washed away and cells were incubated overnight (12-15 hours) in same medium and in co-culture with PMNs in the presence and absence of Fc-folate (3 μM) at a target to effector ratio of 1:60 (T:E). Cell lysis for each cell line was determined by measuring ^{51}Cr released into the supernatant. For each cell line, resulting cpm values in the supernatant from control wells (TNBC cells with no PMNs) were subtracted from all PMN-containing wells. Results are expressed as percent of maximum control lysis where maximum control lysis of each cell line was determined by lysing additional control wells with 2% SDS. Results represent mean $\pm$ SD of $n=2$ experiments performed in triplicate where * $P < 0.01$ compared to samples containing PMN alone. (C) TNBC cells were plated at 1×10^5 cells/well in 96-well black plates overnight in low folate RPMI containing 10% FBS and then labeled with calcein-AM for 45 minutes. Excess dye was washed away with PBS and cells were incubated for 8 hours in co-culture

with PMN and in the presence and absence of Fc-folate (3 μ M) at a target to effector ratio of 1:50 (T:E). After 8 hours, wells were washed and remaining adherent cells were lysed with 2% SDS and detected by measuring calcein fluorescence (ex: 485/20 and em: 528/20). Cell viability for each cell line is expressed as percent of control where control represents the TNBC cells without PMN treatment. Results are the mean $\pm$ SEM of n=3 independent experiments performed in triplicate where *P<0.01 compared to samples containing PMN alone.

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