Interferon-γ Promotes Antibody-mediated Fratricide of Acute Myeloid Leukemia Cells*

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Acute myeloid leukemia (AML) is characterized by the proliferation of immature myeloid lineage blasts. Due to its heterogeneity and to the high rate of acquired drug resistance and relapse, new treatment strategies are needed. Here, we demonstrate that IFNγ promotes AML blasts to act as effector cells within the context of antibody therapy. Treatment with IFNγ drove AML blasts toward a more differentiated state, wherein they showed increased expression of the M1-related markers HLA-DR and CD86, as well as of FcγRI, which mediates effector responses to therapeutic antibodies. Importantly, IFNγ was able to up-regulate CD38, the target of the therapeutic antibody daratumumab. Because the antigen (CD38) and effector receptor (FcγRI) were both simultaneously up-regulated on the AML blasts, we tested whether IFNγ treatment of the AML cell lines THP-1 and MV4-11 could stimulate them to target one another after the addition of daratumumab. Results showed that IFNγ significantly increased daratumumab-mediated cytotoxicity, as measured both by 51Cr release and lactate dehydrogenase release assays. We also found that the combination of IFNγ and activation of FcγR led to the release of granzyme B by AML cells. Finally, using a murine NSG model of subcutaneous AML, we showed that treatment with IFNγ plus daratumumab significantly attenuated tumor growth. Taken together, these studies show a novel mechanism of daratumumab-mediated killing and a possible new therapeutic strategy for AML.

Acute myeloid leukemia (AML)3 is the most common type of acute leukemia in adults and affects over 20,830 people each year (1, 2). AML is a hematologic malignancy characterized by a proliferation of myeloid precursors (“blasts”), which infiltrate the bone marrow, blood, and other tissues (3, 4). Despite the existence of multiple biologically distinct subtypes of AML, the current methodology of treatment includes a regimen of chemotherapy and stem cell transplant (5, 6). Allogeneic hematopoietic stem cell transplantation can be curative for certain patients with AML; however, very few patients are candidates for this procedure (7, 8). Patients over 60 years of age have a worse prognosis due to both chemoresistance and intolerance to intensive chemotherapy, with a median survival of 5–10 months (3, 5, 9, 10). Hence, there is an urgent need for the development of safer and more effective therapeutics for AML.

Monoclonal antibodies are being utilized as a treatment for many different types of cancer and are being actively pursued as a treatment for AML (11, 12). Perhaps the most well known antibody in clinical use for AML was the toxin-conjugated anti-CD33 antibody, gemtuzumab ozogamicin (Mylotarg®). This took advantage of the rapid internalization of CD33 upon antibody binding, thereby delivering the toxin into CD33-expressing cells. However, it was withdrawn from the market due to toxicity issues (13, 14). Today, other CD33-targeting drug-antibody conjugates, such as SGN-CD33A and Fc-engineered anti-CD33 antibodies, are being studied in AML (1, 12, 15). The targeting of FcγRI has similarly been proposed, especially after the finding that IFNγ could increase the expression of the high affinity Fcγ receptor, FcγRI (16–19). Recently, a study was completed using a monoclonal antibody to CD123 that has been humanized, affinity-matured, and Fc-engineered for increased affinity toward CD16 (FcγRIIA), which showed an effect against AML both in vitro and in vivo in an environment with adequate NK cell function (20).

CD38 is a transmembrane glycoprotein expressed in many different cells, including lymphocytes (21–24). The anti-CD38 monoclonal antibody daratumumab has shown a favorable safety profile and encouraging efficacy in patients with refractory multiple myeloma (25–27), and the anti-CD38 SAR650984 is being examined as a treatment for CD38+ hematological malignancies, including AML (clinicaltrials.gov registration NCT01084252). Here, we have found that treatment of AML cell lines and primary AML apheresis samples with IFNγ leads...
to the up-regulation of M1-related markers and of the daratumumab target CD38. IFNγ also induced AML cell fratricide in vitro and reduced tumor growth in vivo, an effect that was significantly enhanced by the addition of anti-CD38. Interestingly, IFNγ also led to FcγR-mediated granzyme B production in AML cell lines. These results suggest that IFNγ can cause the AML cells themselves to become immune effectors and that IFNγ plus anti-CD38 antibody may be an effective treatment for AML.

Results

**IFNγ Promotes an M1-related Phenotype in AML Cells—** Myeloid cells within the context of tumors commonly display M2-like characteristics, which serve to promote tumor growth and survival (28, 29). Here, we tested whether treatment with IFNγ could lead to a shift toward an M1-like phenotype. M1 macrophages can be identified by many different phenotypic markers, including CD80/B7-1, CD86/B7-2, HLA-DR, and NOS2 (28, 30). To test this, we treated AML cell lines MV4-11, MOLM-13, OCI-AML3, and THP-1 for 24 or 48 h and primary AML samples for 18 h, with or without 10 ng/ml IFNγ. Levels of CD86/B7–2 (T-cell co-activator molecule), NOS2, and HLA-DR were measured using qPCR. CD80/B7-1, which works in tandem with CD86/B7-2 as a T-cell co-activator, is expressed at low levels on most M4/M5 AML cells (31). In agreement with this, we found little to no CD80 transcript in the four AML cell lines tested (data not shown).

Results showed that IFNγ significantly increased the transcript of CD86 in all cell lines except THP-1, which showed a strong trend toward significance (Fig. 1A). In primary AML samples, CD86 increased after 18-h IFNγ treatment (Fig. 1B). We then verified that these increases occurred at the cell surface using flow cytometry, with results showing that treatment with IFNγ for 24 h (MV4-11, OCI-AML3, and THP-1) or 48 h (MOLM-13) led to increases in CD86 expression (Fig. 1C). This was recapitulated in primary AML apheresis samples (Fig. 1D). Similarly, IFNγ significantly increased transcript and surface expression of HLA-DR in primary AML apheresis samples (Fig. 1, E and F, respectively), although NOS2 was variable (data not shown).
IFNγ Increases FcγRI Expression and Phagocytic Ability in AML Cells—Because we observed that IFNγ is able to enhance the expression of M1-related markers, we hypothesized that IFNγ could also enhance Fcγ receptor functions, such as phagocytosis. It has previously been shown that IFNγ could increase the expression of FcγRI in AML cells, which led at one point to the exploration of IFNγ treatment combined with drug-conjugated anti-FcγRI antibody as a potential therapy for AML (17).

To assess the effects of IFNγ on phagocytic ability, cell lines were treated with 10 ng/ml IFNγ for either 24 h (MOLM-13, OCI-AML3, and THP-1) or 48 h (MV4-11, which appeared to show a delayed response to IFNγ). Phagocytosis was counted via microscopy in a blinded fashion. The phagocytic index represents the number of red blood cells ingested by 100 AML cells for each cell line. *, p ≤ 0.05. Error bars, S.D.

FIGURE 2. IFNγ increases FcγRI expression and phagocytic ability in AML cells. AML cell lines MOLM-13, MV4-11, OCI-AML3, and THP-1 (n ≥ 3 separate experiments each) and primary AML apheresis samples were treated with or without 10 ng/ml IFNγ for 18 h (qPCR) or for 24 h (flow cytometry). A–D, FcγRI expression in AML cell lines (A) and primary AML apheresis samples (B, n = 12 donors) was measured by qPCR. FcγRI expression in AML cell lines (C) and primary AML apheresis samples (D, n = 3 donors, representative histogram shown; inset bar graph depicts all donors) was measured by flow cytometry. E, AML cell lines were treated with or without 10 ng/ml IFNγ for 24 h (MV4-11 cells for 48 h) and then incubated with opsonized sheep red blood cells. Phagocytosis was counted via microscopy in a blinded fashion. The phagocytic index represents the number of red blood cells ingested by 100 AML cells for each respective cell line. F, primary AML apheresis samples (n = 5 donors) were treated with or without 10 ng/ml IFNγ for 24 h and then incubated for 60 min with opsonized sheep red blood cells. Phagocytosis was counted via fluorescence microscopy in a blinded fashion. The phagocytic index represents the number of red blood cells ingested by 100 AML cells for each donor. *, p ≤ 0.05. Error bars, S.D.
IFNγ Promotes AML Cell Fratricide

We next tested whether IFNγ would have an effect on CD38 expression. We treated the AML cell lines MOLM-13, MV4-11, OCI-AML3, and THP-1, as well as primary AML apheresis samples, with or without 10 ng/ml IFNγ and measured the expression of CD38 using qPCR (18-h treatment) and flow cytometry (24-h treatment). Results showed that IFNγ treatment led to an increase in CD38 transcript in cell lines (Fig. 3A) and primary AML apheresis samples (Fig. 3B). Surface expression also increased for cell lines and primary AML apheresis samples (Fig. 3, C and D, respectively). No changes in CD33 were observed (data not shown). Hence, not only does IFNγ promote increased FcγR expression and function in AML cells; it also increases the expression of a candidate antibody target, CD38. Next, we tested which concentration of IFNγ was required for this up-regulation of CD38, primary AML apheresis samples were treated for 18 h with 0–10 ng/ml IFNγ, and CD38 transcript was measured via qPCR. Results showed trends toward an increase at 0.5 ng/ml but a statistically significant increase at only 10 ng/ml (Fig. 3E).

IFNγ-mediated CD38 Up-regulation Requires p38, NF-κB, and JAK/STAT—Previous studies have shown increased expression of CD38 by IFNγ in chronic lymphocytic leukemia to be JAK/STAT and T-bet-dependent (20). To determine which downstream signaling pathways were required for the up-regulation of CD38 seen with IFNγ treatment, primary AML apheresis samples were pretreated with inhibitors for ERK, PI3K, p38 MAPK, JAK/STAT, and NF-κB. Results showed that IFNγ-mediated up-regulation of CD38 was prevented by the inhibitors for JAK/STAT, p38, and NF-κB (Fig. 4, A–C, respectively). CD38 did not seem to be regulated by ERK (Fig. 4D) or PI3K (data not shown). Western blots with phospho-specific antibodies were performed to verify the efficacy of the inhibitors (data not shown).

IFNγ Enhances Antibody-mediated Fratricide in AML Cells—Results have shown that IFNγ treatment of AML cells promoted their shift toward an M1-related phenotype, enhanced the expression and function of FcγRI, and increased the expression of an antigen target for antibody therapy. Next, we tested the ability of IFNγ to promote antibody-mediated killing within pools of AML cells, a phenomenon termed fratricide (32, 33). We treated THP-1 and MV4-11 cells for 48 h with or without 10 ng/ml IFNγ and then split each group of samples in half to be labeled with either a red or green dye. Green-stained samples within each cell line were opsonized with anti-CD38 antibody on ice, washed with media, and then incubated with their corresponding non-opsonized red cells for 3 h. Conjugate formation was scored using fluorescence microscopy, with non-antibody-mediated conjugates represented as red-red (RR) interactions and antibody-mediated conjugates represented as red-green (RG) plus green-green (GG) (Fig. 5). Results showed that IFNγ led to increased antibody-mediated conjugates for both the THP-1 (Fig. 5A) and MV4-11 (Fig. 5B) cell lines.

To further quantify the amount of antibody-mediated fratricide, we measured cytotoxicity using a 51Cr release assay. Cells were treated with or without 10 ng/ml IFNγ, loaded with 51Cr, opsonized with either anti-CD38 or control IgG, and then incubated for 48 h. Results showed that there was significantly greater antibody-mediated 51Cr release in samples treated with IFNγ within both the THP-1 and MV4-11 sets (Fig. 5C, left and right, respectively). In fact, the combination of anti-CD38 and IFNγ seemed to have a synergistic effect on cytotoxicity, suggesting that the effectiveness of anti-CD38 was dependent on IFNγ-mediated up-regulation of the CD38 antigen.

Granymes are serine proteases that have been shown to be important effectors of natural killer cell and cytotoxic T cell responses (34). We have recently shown that monocytes treated with TLR8 agonists produced granzyme B and that this contributed to antibody-dependent cellular cytotoxicity (ADCC) (35), so we tested whether IFNγ treatment plus FcγR activation might elicit granzyme B from AML cells. We pretreated THP-1 and MV4-11 cells with or without IFNγ for 18 h and then incubated cells with or without immobilized IgG for 24 h. Results showed that this combination led to significant increases in granzyme B production in both THP-1 (Fig. 5D) and MV4-11 (Fig. 5E) cells.

Next, to further confirm cytotoxicity and to extend the studies to all four AML cell lines, we treated MOLM-13, MV4-11, OCI-AML3, and THP-1 for 24 h with or without 10 ng/ml IFNγ and incubated them for an additional 3 h with anti-CD38 antibody. Cell death was measured via lactate dehydrogenase (LDH) release. As shown in Fig. 6A, IFNγ significantly enhanced antibody-mediated killing within each respective AML cell line. To test whether this was due to a toxic effect of anti-CD38, we incubated the AML cell lines on immobilized anti-CD38 for the same length of time and found that LDH release was not affected (data not shown). This again suggests

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that the anti-CD38 antibody led to cell-against-cell killing, which was triggered and enhanced by IFNγ pretreatment.

IFNγ Enhances Anti-CD38 Therapy in Vivo—We next tested whether IFNγ-induced antibody-mediated fratricide could occur in vivo. To do this, we injected MV4-11 cells subcutaneously into the flanks of NSG mice, which are deficient in B, T, NK, and dendritic cell activity (36), whereas the parent NOD/scid mice show defects in macrophage maturation (37). After 1 week (to permit tumor growth), mice were treated with PBS, anti-CD38 antibody, IFNγ, or a combination of anti-CD38 and IFNγ. Both the rate of tumor growth and the final tumor volumes were measured. Results showed that neither of the single-agent treatments had an effect on the rate of growth, but the combination of anti-CD38 and IFNγ led to a significantly reduced growth rate (Fig. 6B, \( p < 0.01 \)). Final tumor sizes (day 17) were also compared, and results showed that both the IFNγ-treated and the combination-treated groups had significantly smaller tumors (\( p < 0.01 \) and \( p < 0.001 \), respectively; Fig. 6B). These results suggest that the combination treatment was superior, although single-agent IFNγ also showed an effect on final tumor size. Because NSG mice are severely immunodeficient, these results suggest that at least a large portion of the antitumor effects seen with IFNγ and daratumumab were mediated by the AML cells themselves.

Discussion

Herein, we have shown that treatment of AML blasts with IFNγ enables them to perform antibody-mediated fratricide. IFNγ treatment of AML cells was accompanied by increased expression of M1-related markers, resulting in an activated phenotype and increased expression of CD38, the target for the daratumumab. In addition, IFNγ significantly enhanced the IgG-mediated production of granzyme B, suggesting that the AML cells had taken on bona fide effector functions.

Previous work has shown that myeloid leukemia cells could be induced to differentiate by external signals. For example, all-trans-retinoic acid can cause acute promyelocytic leukemia cells to differentiate (37), and an agonistic antibody for the thrombopoietin receptor has been shown to cause AML cells to differentiate into active effector cells with characteristics of both dendritic and natural killer cells. These cells expressed substantial levels of granzyme B, perforin, and IFNγ and were
capable of attacking one another via contact with needle-like filopodia (38).

The potential for IFNγ to stimulate effector functions on AML cells was of particular interest to us in light of its known role in up-regulating FcγRI expression (17). Attempts to exploit this have been made by linking anti-FcγRI to ricin, and this was tested in vitro and in vivo (17). However, although FcγRI is known to mediate cytotoxicity against tumor cells (39), IFNγ treatment for the purpose of stimulating antibody-mediated fratricide had not been fully explored. It is likely that the success of this strategy was not due solely to the up-regulation of FcγRI, however, because we also observed changes in M1-related markers. Such non-FcγR-mediated effects would be expected (and desired). Indeed, STAT1 and STAT3 were among the top responders to treatment with a pleiotropic thrombopoietin agonist antibody that elicited a shift toward DC/NK phenotypes in AML cells (38). Results from the present study suggest that, although IFNγ alone may not drive the full differentiation of AML cells as seen by Yea et al. (38), it is sufficient to promote antibody-mediated fratricide and enhance efficacy of antibody therapy. Traditionally, antibodies exert antitumor activity through antibody-dependent cellular cytotoxicity or complement-mediated cytotoxicity. Here we show that combination treatment with IFNγ resulted in a novel mechanism of action of daratumumab against AML blasts. As such, IFNγ may represent a broader treatment that could be tailored to a number of different antibody-based strategies.

One unexpected finding in the present study was that IFNγ led to the up-regulation of CD38 on AML cells. Although CD38 is known for its pro-survival signaling (40, 41), higher levels of CD38 could also make it more targetable by therapeutic antibodies, such as daratumumab. Indeed, certain agents are already being investigated for their ability to modulate CD38 expression for this purpose. For example, it was recently shown that all-trans-retinoic acid (ATRA) increased CD38 expression in multiple myeloma cell lines and in primary patient samples, significantly enhancing the effects of daratumumab in vitro and in vivo (42). Within the context of AML, ATRA is already being used as a therapy in the M3 subtype, acute promyelocytic leukemia, due to its ability to promote terminal differentiation of malignant cells. Although ATRA does not show single-agent efficacy in other AML subtypes (43, 44), its use with daratumumab is clearly a potential treatment strategy that should be explored.

IFNγ (IFNγ1b, Actimmune®, Horizon Pharma, Inc.) is an approved treatment for preventing infections in chronic granulomatous disease and for delaying severe malignant osteopetrosis (45–48). IFNγ is also being evaluated in combination with checkpoint inhibitors (nivolumab) in solid tumors (NCT02614456). The preclinical data presented here provide a strong rationale for combination of IFNγ and daratumumab in patients with acute myeloid leukemia. For example, Toll-like receptor agonists have been previously shown as effective enhancers of immune responses. In fact, the TLR7 agonist imiquimod is being used as a treatment for superficial basal cell carcinoma and
HPV infection (49, 50). NK cells express both TLR7 and TLR8 and have been shown to produce IFNγ when stimulated by TLR7/8 ligands (51). TLR agonists combined with antibody therapy may stimulate a more localized production of IFNγ, which could be of benefit where there is binding of therapeutic antibody.

In summary, we have found that IFNγ can stimulate AML blasts to become effector cells and target one another in an antibody-dependent manner. Hence, strategies to enhance IFNγ production, including exogenous administration, may offer an effective way to improve the efficacy of antibody therapy for AML.

Experimental Procedures

Cell Culture—The AML cell lines used in this study (MOLM-13, MV4-11, OCI-AML3, and THP-1) were purchased from the ATCC and cultured according to ATCC recommendations. Cells were maintained below $1 \times 10^6$ cells/ml in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Grand Island, NY), 2 mM L-glutamine (Invitrogen), and penicillin/streptomycin (56 units/ml/56 μg/ml; Invitrogen) at 37 °C in an atmosphere of 5% CO₂.

Primary Cells—White blood cells apheresed from AML patients were obtained after written informed consent in accordance with the Declaration of Helsinki under a protocol approved by the Ohio State University institutional review board. Cells were stored in liquid nitrogen in 20% FBS and 10% DMSO until needed for experiments. At the time of the experiment, cells were thawed at 37 °C and incubated in RPMI 1640 medium (Gibco) supplemented with 20% FBS, 2 mM L-glutamine (Invitrogen), and penicillin/streptomycin (56 units/ml/56 μg/ml; Invitrogen) at 37 °C in an atmosphere of 5% CO₂ for 1 h.
Cells were then centrifuged and incubated in RPMI 1640 medium (Gibco) supplemented with 20% FBS, 2 mM L-glutamine (Invitrogen), and penicillin/streptomycin (56 units/ml/56 μg/ml; Invitrogen) and were either left untreated or treated with 10 ng/ml recombinant human IFNγ (R&D Systems, Minneapolis, MN) and incubated for 18 h at 37 °C. The next day, cells were counted using trypan blue exclusion and used for assays.

**Cytokines and Antibodies**—Recombinant human IFNγ (R&D Systems) was added to cell cultures at a concentration of 10 ng/ml. For the LDH assays, anti-human CD38 (clone HIT2; BD Biosciences) was used to coat cells. Briefly, cells were incubated with 10 μg/ml antibody for 1 h on ice, washed once with PBS, and resuspended in medium. Daratumumab was used for ADCC (20 μg/ml), conjugate studies (10 μg/ml), and in vivo experiments (1 μg/g mouse weight) and was supplied by commercial sources (Ohio State University, Columbus, OH).

For flow cytometry, unconjugated mouse anti-human CD64 (clone 32.2) with an FITC goat anti-mouse secondary antibody (Invitrogen), anti-human CD38 conjugated to FITC (clone HIT2; BD Biosciences), anti-human CD86 conjugated to phycoerythrin (clone 2331 (FUN-1); BD Biosciences), and anti-human CD80 conjugated to APC-H7 (clone L307.4; BD Biosciences) were used. Briefly, cells were incubated with 1 μg of antibody at a concentration of 1 × 10⁶ cells/ml for 30 min at 4 °C. Cells were washed twice with FACS buffer (PBS, 0.09% sodium azide, 10% FBS). Secondary only (anti-human-CD64) and isotype control (all others) labeling was done in parallel to control for nonspecific staining. Samples were analyzed using an LSRII flow cytometer (BD Biosciences) and FlowJo software (FLOWJO, LLC, Ashland, OR).

**Inhibitors**—The PI3K inhibitor LY294002 (used at 20 μM) and ERK inhibitor U0126 (used at 25 μM) were purchased from Calbiochem (Billerica, MA). The p38 inhibitor, SB203580 (used at 5 μM), was purchased from Sigma. The NF-κB inhibitor, BAY 11-7085 (used at 5 μM), was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). The JAK1/2 inhibitor, ruxolitinib (used at 50 nM), was purchased from Selleck Chemicals (Houston, TX).

**Real-time PCR**—Total RNA was isolated using TRIzol reagent (Invitrogen) and chloroform extraction followed by DNase treatment (Invitrogen). RNA was reverse transcribed and subjected to quantitative real-time PCR using Power SYBR Green Master Mix (Applied Biosystems, Grand Island, NY). The following primers were used: GAPDH (forward primer, 5'-ATT CCC TGG ATT GTG AAA TAG TC-3'; reverse primer, 5'-ATTAAAGTCCACCGCTTCTGTA-3'), FcγRI (CD64) (forward primer, 5'-GGCAAGTGAGCACCAAAGGCAG-3'; reverse primer, 5'-GCTGGGAGTCAGCTCGGATG-3'), CD86 (forward primer, 5'-GGTCTGGCTGGTC-3'; reverse primer, 5'-TCCTGGCARAAGTCGAAG-3'), CD80 (forward primer, 5'-GGTCTGGCTGGTC-3'; reverse primer, 5'-TCCTGGCARAAGTCGAAG-3'), qPCR primers for HLA-DQ (forward primer, 5'-ATTAAAGTCCACCGCTTCTGTA-3'; reverse primer, 5'-GCTGGGAGTCAGCTCGGATG-3'), CD86 (forward primer, 5'-GGTCTGGCTGGTC-3'; reverse primer, 5'-TCCTGGCARAAGTCGAAG-3'), GAPDH, and SDF-1 (Hs.PT.58.27881121) were purchased from Integrated DNA Technology (San Diego, CA). GAPDH was used for normalization of the genes of interest. Data were presented as mean relative copy number for at least three separate experiments using relative copy number = 2⁻ΔCt × 100 (52), where ΔCt is the Ct of target - Ct of GAPDH.

**Lactate Dehydrogenase Assay**—Antibody-coated (anti-CD38) or uncoated cells (5 × 10⁴/100 μl of medium) that were pretreated for 24 h with 10 ng/ml IFNγ or left untreated were plated in quadruplicate in a 96-well plate. IFNγ (10 ng/ml) was added to appropriate wells and then incubated for 18 h at 37 °C. The CyTox 96 non-radioactive cytotoxicity assay (Promega, Madison, WI) was used to measure released LDH from cells.

**FIGURE 6. IFNγ enhances anti-CD38 therapy in vivo.** A, AML cell lines MOLM-13, MV4-11, OCI-AML3, and THP-1 (n = 3 separate experiments each) were treated for 24 h with or without 10 ng/ml IFNγ and then incubated for 3 h with or without 10 μg/ml anti-CD38 antibody (CD38). IFNγ was added to the respective treatments for 18 h, and LDH assays were performed to measure cytotoxicity. *, p ≤ 0.05 versus untreated (UT), B, NSG mice were subcutaneously injected with 2.5 × 10⁶ MV4-11 cells and then treated with either PBS, anti-CD38 antibody, IFNγ, or a combination of anti-CD38 and IFNγ (n = 6/group). Tumor growth rate as well as final tumor volumes were measured. *, p ≤ 0.05 versus PBS control at day 17 for rate of tumor growth. Error bars, S.D.
**IFNγ Promotes AML Cell Fracture**

This assay was performed as per the manufacturer’s instructions. After an 18-h incubation, plates were centrifuged at 250 × g for 4 min, supernatants were collected and transferred to an ELISA plate, and then the supernatants were incubated with LDH substrate for 30 min. Stop solution was added, and the plates were read on a plate reader at a wavelength of 490 nm. Percentage cytotoxicity was calculated as (experimental absorbance/LDH maximum release) × 100. The assay was repeated at least three times for each cell line.

**Phagocytosis**—Phagocytosis assays were performed as described previously with minor adaptations for the experimental requirements of this study (53). Briefly, sheep red blood cells (SRBCs; Colorado Serum Company, Denver, CO) were labeled with PKH26 fluorescent cell membrane dye (Sigma) and then opsonized with anti-SRBC antibody (Sigma). SRBCs were added to the respective AML cell lines (treated with IFNγ for 24 or 48 h) or primary AML apheresis samples (treated with IFNγ for 24 h), gently pelleted by slow centrifugation, and then incubated at 37 °C for 1 h. Non-phagocytosed SRBCs were lysed with red blood cell lysis buffer (eBioscience, San Diego, CA) at room temperature for 10 min and washed with PBS before fixation with 4% paraformaldehyde. The SRBCs ingested by the AML cells were counted in a blinded fashion using fluorescence microscopy, with three separate such counts per condition. For each set of counts, 100 phagocytes/condition were examined. The phagocytic index is defined as the total number of SRBCs ingested by 100 phagocytes.

**Conjugate Formation Assay**—Conjugate formation assays were performed using MV4-11 and THP-1 cell lines. Cells were treated with or without 10 ng/ml IFNγ and incubated at 37 °C for 48 h. Following incubation, samples were washed twice with PBS and split evenly into two tubes, one-half to be stained red and the other to be stained green. Staining was done using the CellVue® claret far red fluorescent cell linker kit and PKH67 green fluorescent cell linker kit, both from Sigma-Aldrich, according to the manufacturer’s instructions. Following this, selected cell groups were opsonized by incubating with 10 µg/ml anti-CD38 antibody on ice for 1 h, followed by two washes with complete medium. For the assay, red and green cells were combined into 1 ml of medium and incubated at 37 °C for 1 h. Samples were washed in PBS three times and fixed in 4% paraformaldehyde. 30 random images were taken using a fluorescence microscope, and conjugates were counted in a blinded fashion.

**Murine Model of Antibody Therapy**—Female non-obese diabetic severe combined immunodeficient-γ (NSG) mice were purchased from Jackson ImmunoResearch Laboratories (Bar Harbor, ME). MV4-11 cells (0.25 × 10^6 cells resuspended in PBS) were subcutaneously injected into the right flank of 6-week-old NSG mice and allowed to grow for 7 days (36). Intrapertitoneal injections with PBS vehicle, IFNγ (3,000 units/mouse), daratumumab (anti-CD38 antibody; 1 µg/g mouse weight), or IFNγ + daratumumab (anti-CD38 antibody) were administered twice per week for 2 weeks, with tumor measurements recorded on each treatment day in a blinded fashion. Tumor volumes were calculated as π/6(length × width × height) (54). All in vivo experiments were performed in strict accordance with guidelines set by the institutional animal care and use committee, under an approved protocol.

**ADCC**—ADCC assays were done as described previously (55). In brief, THP-1 and MV4-11 cells were treated with or without IFNγ (10 µg/ml), loaded with 51Cr, coated with anti-CD38 antibody or IgG antibody, and plated in V-bottom 96-well plates. After 48 h of incubation, levels of 51Cr in supernatants were measured using a γ counter. The percentage cytotoxicity was calculated as (sample — minimum)/(maximum — minimum) × 100, where minimum consisted of untreated cells not incubated with antibody and maximum was measured as cells that had been lysed with 10% SDS.

**Statistical Analyses**—The qPCR and phagocytosis data were analyzed using paired Student’s t tests. To evaluate the association of IFNγ-mediated changes in phagocytosis and changes in FcγRI expression (Table 1), we first transformed the changes relative to baseline by using log transformation to fit the normality assumption, followed by performing Pearson correlation analysis. Cell death assays using released LDH were analyzed by mixed effect modeling, incorporating repeated measures for each sample. Tumor volumes from the in vivo study were first baseline-subtracted and then analyzed by mixed effect modeling, incorporating repeated measures for each tumor. Tumor growth rates and final tumor volumes were both compared. SAS 9.4 (SAS Inc., Cary, NC) was used for analyses.

**Author Contributions**—K. F., E. L. M., B. F. R., H. F., R. S., G. S., S. E., P. M., N. J. B., and G. M.-R. designed and performed experiments and collected and summarized data; X. M. performed statistical analyses and designed experiments; J. S. B. provided critical human samples and designed experiments; S. V., D. M. B., W. E. C., and J. C. B. designed experiments, provided reagents and helped write the paper; J. P. B. and S. T. designed experiments, analyzed results, and wrote the paper along with K. F., B. F. R., and S. V.; all authors reviewed and revised the manuscript and approved the final version.

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**References**

IFNγ Promotes AML Cell Fratricide


