NK Cell-Mediated Antitumor Effects of a Folate-Conjugated Immunoglobulin Are Enhanced by Cytokines


Abstract

Optimally effective antitumor therapies would not only activate immune effector cells but also engage them at the tumor. Folate conjugated to immunoglobulin (F-IgG) could direct innate immune cells with Fc receptors to folate receptor–expressing cancer cells. F-IgG bound to human KB and Hela cells, as well as murine L1210F, a folate receptor (FR)–overexpressing cancer cell line, as determined by flow cytometry. Recognition of F-IgG by natural killer (NK) cell Fc receptors led to phosphorylation of the ERK transcription factor and increased NK cell expression of CD69. Lysis of KB tumor cells by NK cells increased by about 5-fold after treatment with F-IgG, an effect synergistically enhanced by treatment with IL2, IL12, IL15, or IL21 (P < 0.001). F-IgG-coated targets also stimulated FcR-mediated monocyte effector functions. Studies in a murine leukemia model confirmed the intratumoral localization and antitumor activity of F-IgG, as well as enhancement of its effects by IL12 (P = 0.05). The antitumor effect of this combination was dependent on NK cells and led to decreased tumor cell proliferation in vivo. Thus, F-IgG can induce an immune response against FR-positive tumor cells that is mediated by NK cells and can be augmented by cytokine therapy.

Introduction

Folate is a water-soluble B vitamin that functions as a cofactor in various cellular metabolic processes, such as nucleic acid synthesis and protein biosynthesis. Green vegetables, fresh fruits, and fortified foods are sources of folate (1). Cellular uptake of folate is mediated by at least three distinct transporters, which include the proton-coupled high-affinity folate transporter, the reduced folate carrier (RFC), and the folate receptor (FR; ref.2). FR expression is normally low and limited to specific tissues, such as the choroid plexus, epididymis, and kidney. In normal
polar epithelial cells, the FR is predominantly located on the luminal (nonvascular) side of the cell. Thus, the FR expressed on normal cells is largely inaccessible to circulating folate or folate conjugates (5). The oncogenic potential of the FR was first observed by Sidney Farber in 1948, who reported that an accelerated leukemic process was present in children treated with derivatives of folate (6). The FR is overexpressed on the surface of malignant cells, such as cancers of the cervix, breast, testes, and ependymal brain tumors (2, 7–9). This accessibility, relative to that of normal cells, makes the FR an attractive therapeutic target. Once a folate conjugate is bound, it may be taken up via endocytosis while a fraction remains engaged with the FR on the cell surface (5). These parameters are thought to create a favorable toxicity profile for folate-conjugated antitumor compounds (2).

Natural killer (NK) cells are bone marrow–derived lymphocytes that can lyse malignant cells with altered expression of major histocompatibility antigens. NK cells express killer cell immunoglobulin receptors (KIR), cellular adhesion molecules, and multiple cytokine receptors (e.g., receptors for IL12, IL15, and IL18). NK cells also produce several cytokines with antitumor effects (i.e., IFNγ, TNFα, and MIP-1α) and possess the ability to lyse malignant cells using cytolytic granules that contain perforin and granzymes. NK cells also express the FcyRIIIa (CD16) receptor, a low-affinity activating receptor that recognizes the constant (Fc) region of IgG. This receptor is critical in mediating antibody-dependent cellular cytotoxicity (ADCC) against antibody-coated targets (10).

A folate-conjugated immunoglobulin (F-IgG) that is able to bind specifically to FR-bearing tumor cell lines was tested for its ability to mediate ADCC and cytokine production by NK cells. We also characterized the antitumor and immune effects of the F-IgG conjugate when used in combination with NK-activating cytokines that activate Fc receptor (FcR)-bearing NK cells.

Materials and Methods

Human and murine F-IgG synthesis

F-IgG (derived from all classes of IgG) and F-IgG-fluorescein isothiocyanate (FITC) were synthesized as described (11). Three folates per IgG were utilized for these studies. Modified human or murine IgG1 molecules were used as controls (C-IgG). The molecular weight of the F-IgG construct is approximately 151 kDa.

Cell lines

The FR+ human cell lines KB (gift from Dr. Philip S. Low, Purdue University in 2006), HeLa, and F01 (human cervical and melanoma; ATCC obtained in 2002); OSU-NB and OSU-CLL (normal B and CLL cell lines, derived by J. Byrd from a CLL patient in 2012); and L1210JF (murine leukemia, gift from Robert J. Lee, Ohio State University in 2007) as well as the FR-negative cell line A549 (human alveolar adenocarcinoma; ATCC obtained in 2006) were propagated in folate-free RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic. The identity of these cell lines was not confirmed with TRizol Reagent using the RNeasy Mini Kit (Qiagen) following the manufacturer’s protocol. RNA was converted to complementary DNA by reverse transcription and used as a template for RT-PCR using folate receptor alpha primers with the internal control 36B4 (14, 15).

Confocal microscopy and intratumoral flow cytometric analysis

KB tumor cells were treated with C-IgG or F-IgG–FITC for 30 minutes at 37°C and collected at the designated time points. Cells were washed with flow buffer (5% FBS in PBS), fixed in 1% formalin in PBS, mounted on 25 × 75 × 1 mm slides (Erie Scientific) and stained with DAPI for nuclear identification (Vector Labs) prior to analysis by immunofluorescence (IF) microscopy. Intracellular analysis of NK cell ERK phosphorylation was also assessed following coculture with KB tumor cells. NK cell activation was assessed 48 hours after coculture via FACS analysis with CD56–phycoerythrin (PE), CD158–allophycocyanin (APC), CD57–FITC, NK22D-PE (Beckman Coulter), CD65–APC, CD3-V50, CD16–FITC, NK22D-APC, CD69–phycoerythrin-cyanin 7 (Pe-Cy7) and KIR/NKAT2-FITC, CD57–FITC, NKG2A–PE (Beckman Coulter), CD56–APC, CD335–APC antibodies (BD Biosciences). In murine studies, IF microscopy of tumor sections was performed (16). Sections were analyzed on an Olympus Fluoview 1000 laser scanning confocal microscope. In murine studies with C-IgG- and F-IgG–FITC, tissues were harvested in the dark at indicated times. Tumors were dissociated (17) and stained with FR-R-APC (R&D Systems). FR+ /FITC+ populations were assessed using a Becton Dickinson FACS Calibur flow cytometer (Becton-Dickinson; ref. 16).

NK cell FcR stimulation assays and immunoblot analysis

Plates were coated with 50 μg/mL control IgG (C-IgG) or F-IgG overnight at 4°C and then plated with NK cells. After 15 minutes at 37°C, cells were lysed in TN1 or 0.5% Brij-98 lysis buffer and probed with anti-phospho-ERK or ERK primary antibodies (Cell Signaling Technology), FR (FL-257, Santa Cruz Biotechnology) or β-actin antibodies (Sigma; ref. 18).

Cytotoxicity assays

For ADCC assays, purified NK cells or monocytes were plated with medium supplemented with IL2 (10 ng/mL), IL12 (0.5 ng/mL or 10 ng/mL), IL15 (10 ng/mL), or IL21 (10 ng/mL). F-IgG or C-IgG–treated (100 μg/mL)51Cr-labeled target cells were added at various effector:target (E:T) ratios for 4 hours (NK cells) or 18 hours (monocytes). Supernatants were harvested, and 51Cr was quantified (19).

In vitro coculture assay

KB and A549 cell lines were coated with F-IgG or C-IgG (100 μg/mL) and cocultured with NK cells or monocytes with medium containing IL2, IL12, IL15, IL18, or IL21 (10 ng/mL;...
ref.12). After 48 hours, supernatants were analyzed for IFNγ, MIP-1α, TNFα, and RANTES content by ELISA and utilized in T-cell chemotaxis experiments (20). For MDSC coculture experiments, MDSCs were generated (21) and added to NK cell cocultures at a ratio of 0.5:1 MDSC:NK cell.

Murine tumor model

Mice were placed on a folate-deficient diet (AIN-90G, Dyets) 1 week prior to tumor cell injection (11). Age-matched, female DBA/2NCrl mice (Strain code 026; Charles River) were injected with $10^5$ L1210JF cells subcutaneously (s.c.). For intratumoral localization studies, age-matched, female athymic mice Crl:NU(NCR)−Foxn1nu (Strain code 490; Charles River) were inoculated with $4 \times 10^6$ human KB cells. Once tumors were palpable, mice were treated twice a week intraperitoneally (i.p.) with C-IgG or F-IgG (100 mg/kg), 2.5 ng mIL12 (Wyeth Pharmaceuticals), or the combination. Tumor volumes were assessed three times a week (16). To deplete monocytes/macrophages, 1 mg/kg of clodronate liposomes was administered on the day of tumor implantation and 0.5 mg/kg every 4 days thereafter (22). NK cells were depleted via anti-asialo GM1 (16).

Histologic analysis of murine tissues

Murine tissues were harvested and processed immediately. Tumors were then sectioned and stained and quantified using the ScanScope XT and Positive Pixel Count Algorithm ImageScope software (Aperio Technologies) as described (16, 23).

Statistical analysis

The synergistic effects of F-IgG and cytokines on NK cell–mediated ADCC, IFNγ, or MIP-1α production were tested using linear mixed models to account for the correlated responses from...
Figure 2.
Phospho-ERK upregulation and cytolytic activity of NK cells in response to F-IgG stimulation. A, assessment by immunoblot. Freshly isolated NK cells were plated on immobilized C-IgG or F-IgG at 37°C for 15 minutes. Control conditions consisted of NK cells treated with PBS or PMA (positive control). NK cells were harvested and lysed for immunoblot analysis of phospho-ERK. Total ERK was measured as a loading control, and relative quantification of P-MAPK versus ERK is indicated. B, assessment by intracellular flow cytometry. NK cells were stimulated with media, PMA-, C-IgG-, or F-IgG-coated KB tumor cells for 45 minutes, and levels of phospho-ERK were determined by flow cytometry. (Continued on the following page.)
the same cell types and the same donor cells. In treatment groups (C-IgG, F-IgG, or the combination of C-IgG or F-IgG plus cytokines), E:T ratios were considered as a fixed effect. Ratios and treatment interactions were also included in the model. To control for type I error, gatekeeping strategies were used in each experiment at a statistical significance level of 0.05, we first tested the difference between the combination treatments to the C-IgG treatment group (control) across different E:T ratios. If the difference was statistically significant ($P < 0.05$), then the differences across different E:T ratios were further tested for the combination treatments versus F-IgG or C-IgG, and F-IgG versus C-IgG. The $P$ values for each hypothesis were based on the corresponding interaction contrasts of the fixed-effects parameters estimated from the linear mixed models using the estimate or lsmeans statement in the Proc Mixed procedure. T-cell migration studies were analyzed using a two-way ANOVA with Tukey methods to correct for multiple comparisons. Changes in tumor volume over time were assessed via a linear mixed model for longitudinal data. Tumor volumes were log-transformed in the model, and the differences in tumor volume were calculated at baseline and at the end of each study time points based on a linear mixed model. All data analyses were performed using SAS V9.4 statistical software.

**Results**

**Cell line FR-α expression**

Both the human KB and HeLa tumor cell lines expressed the FR-α transcript, as indicated by RT-PCR (Fig. 1A), but not the A549 cell line. FR protein content was confirmed by immuno-blot analysis in the HeLa, KB, and murine L1210FF cell lines (Fig. 1B). Surface expression of the FR was detected on the KB and L1210FF cell lines as determined by flow cytometry using an F-IgG-FITC-labeled conjugate. The specificity of the interaction between F-IgG and the FR is supported by F-IgG binding inhibition in KB cells pretreated with folate (200 nmol/L; Fig. 1C). In addition, F-IgG-FITC binding was maintained for greater than 24 hours, as assessed by confocal microscopy, whereas C-IgG-FITC did not exhibit this binding (Fig. 1D). No FR was detected on NK cells (data not shown). Based on these experiments, the KB and L1210FF cell lines were utilized for in vitro and murine studies.

**F-IgG activates NK cells**

NK cells can interact with antibody-coated target cells via an activating, low-affinity Fc receptor (CD16), which can activate the MAPK/ERK signaling pathway (12, 19, 20). Stimulation of this pathway is critical for FcR-mediated NK cell lytic function and cytokine production. We hypothesized that binding of human NK cells to immobilized F-IgG would activate this pathway and result in the phosphorylation of ERK. As predicted, NK cells stimulated with F-IgG (or C-IgG) exhibited striking ERK activation at 15 minutes, as compared with the PBS-stimulated cells (Fig. 2A). NK cell ERK phosphorylation was also determined following exposure to F-IgG-coated tumor cells. An increase in the phosphorylation of ERK was observed in NK cells that were cocultured with F-IgG–coated KB tumor cells after 45 minutes of treatment (Fig. 2B). CD69 upregulation on NK cells is associated with a productive anti-tumor response (24). CD69 expression on NK cells increased in response to F-IgG–coated tumor cells in both the CD56 bright and CD56 dim subsets (Fig. 2C). Activated NK cells also decreased CD16 expression when incubated with F-IgG, compared with C-IgG, an indication of Fc receptor occupation (data not shown; ref. 25). No difference in the surface expression of NKG2D, CD57, Nkp46/CD335 (activating receptors), CD158, NKG2A, CD94, or KIR/NKAT2 (inhibitory receptors) was observed (data not shown).

**F-IgG treatment of FR$^\text{+}$ tumor cells enhances lysis**

The effect of F-IgG on NK cell ADCC of FR$^\text{+}$ tumor targets was examined using KB tumor cells as targets in a standard 4-hour ADCC assay with healthy donor human NK cells as effectors (19). NK cell-mediated ADCC with F-IgG coating of targets was significantly enhanced at all E:T ratios and in every donor tested, as compared with C-IgG ($P < 0.0001$; Fig. 2D). F-IgG treatment did not enhance the lysis of the FR-negative cell line A549 (Fig. 2E). The difference in the average percent lysis between F-IgG and C-IgG was significantly greater in the KB cell line as E:T ratios increased ($P < 0.001$; Fig. 2D). This trend was not observed in A549 cells (Fig. 2E).

**Cytokine enhancement of NK ADCC**

Next, several NK cell–activating cytokines were evaluated for their ability to augment NK cell ADCC against F-IgG–treated KB tumor cells. NK cells were pretreated with cytokines (IL2, IL12, IL15, or IL21) and cocultured with F-IgG–coated tumor cells at various E:T ratios in a $^{51}$Cr release assay (19). With every cytokine tested, F-IgG or cytokine-mediated lysis was significantly higher than that of C-IgG treatment alone (all $P < 0.01$). The combination of F-IgG and IL2 (Fig. 3A), IL12 (Fig. 3B), IL15 (Fig. 3C), or IL21 (Fig. 3D) resulted in significantly higher tumor cell lysis than either F-IgG or cytokine treatment alone across all E:T ratios (all $P < 0.001$). A statistically significant synergistic lysis effect was observed following treatment with every cytokine evaluated across different E:T ratios (IL2 $P = 0.0011$, IL12 $P < 0.0001$, IL15 $P = 0.0017$, IL21 $P < 0.0003$). Next, FR-blocking experiments were conducted, in which the KB tumor cells were treated with varying amounts of folate to create high, medium, and low FR availability, and validated by flow cytometry (data not shown). NK cell ADCC was then assessed. As predicted, NK cell killing directly correlated with the level of available FR (Supplementary Fig. S1A). In addition, NK cells from 8 normal donors were genotyped for CD16 variants, and ADCC assays were conducted. The data for one representative F/F (lower affinity—donor 3) and F/V donor (intermediate—donor 7) are shown in Supplementary Fig. S1B.
Figure 3.
F-IgG NK cell-mediated lysis is enhanced by cytokines. The lytic activity of cytokine-activated NK cells was assessed in a standard 4-hour chromium release assay in the presence of C-IgG or F-IgG-coated KB tumor cells. (Continued on the following page.)
IL12 enhances lysis of F-IgG-treated CLL cells

Our group is also interested in immune-based approaches for the treatment of chronic lymphocytic leukemia (CLL). We therefore examined the effects of F-IgG in this context. The expression of the FR in CLL has been described (27) and was validated in patients with CLL by our group (Fig. 3E). The ability of CLL patient NK cells (TIL) to kill F-IgG-coated KB (Fig. 3F, left) or autologous CLL target cells (Fig. 3F, right) was determined in a standard ADCC assay, in which care was taken to remove FR-bound folate from target cells (28). Here, freshly isolated NK TILs from CLL patients mediated significant lysis against both targets and the cytolytic activity of these NK cells was markedly enhanced following stimulation with IL12 (P < 0.0005; Fig. 3F).

IL12 enhances cytokine production and T-cell migration

Costimulation of NK cells via the IL12R and CD16 leads to enhanced production of IFNγ due to colocalization of these receptors at cell membrane lipid rafts and subsequent enhancement of signal transduction (18). We hypothesized that NK cell–activating cytokines would enhance NK cell production of IFNγ following exposure to F-IgG–coated KB tumor cells. An in vitro assay was performed in which purified NK cells were cocultured with F-IgG-coated FR” tumor cells (12), KB (FR”) and A549 (FR”) cells were treated with F-IgG or C-IgG and plated with purified NK cells in the presence or absence of IL2, IL12, IL15, IL18, or IL21. Supernatants were harvested 48 hours later and assayed for IFNγ content. As observed in the ADCC experiments, F-IgG treatment not only resulted in a significant increase in NK cell IFNγ secretion (all P < 0.0001), but also demonstrated a significant interaction with IL2, IL12, IL15, IL18, and IL21 (P < 0.01; Fig. 4A). As the coactivation of IL12R and CD16 can stimulate the secretion of other cytokines, the concentrations of MIP-1α and RANTES were also measured. NK cell production of MIP-1α (Fig. 4B) and RANTES (Fig. 4C) was enhanced following treatment with F-IgG–coated tumor cells and IL12, compared with C-IgG (P < 0.0001). Treatment of the FR” cell line A549 with F-IgG did not enhance NK cell IFNγ production in response to any cytokine (data not shown).

NK cell–derived factors produced in response to immobilized IgG can serve as a powerful stimulus to T-cell chemotaxis (20). As predicted, T-cell chemotaxis was significantly enhanced upon exposure of T cells to coculture supernatants that were obtained following treatment of NK cells with F-IgG–coated tumor cells and IL12, compared with C-IgG (P < 0.01; Fig. 4D). These results provide evidence that coadministration of cytokines with F-IgG can significantly enhance NK cell production of chemokines, which have the ability to recruit T cells to sites of inflammation. Human NK cells stimulated with F-IgG–coated tumor cells plus IL12 increased CD69 expression, a finding that is consistent with dual activation of IL12R and FcR (Fig. 4E).

Induction of monocyte cytolytic activity and secretion of TNFα

Experiments were conducted to determine if F-IgG could enhance the effector functions of monocytes, another immune cell population that is critical to antibody-mediated immune responses (29). Lysis of FR” KB tumor cells by normal monocytes was significantly enhanced by pretreatment of the target cells with F-IgG (P < 0.0001), an effect that was further enhanced by activation of monocytes for 18 hours with IL12 (Fig. 5A). In addition, peripheral blood monocytes (PBMC) secreted significantly large quantities of TNFα when exposed to plate-bound (Fig. 5B) and tumor-bound F-IgG (Fig. 5C), as compared with controls (P < 0.001). Monocytes also secreted IL12 in response to F-IgG–coated KB tumor cells (Fig. 5D; P < 0.004). The role of monocytes in mediating the effects of F-IgG plus IL12 therapy was examined via the use of myeloid-depleting clodronate liposomes (22). Mice treated with clodronate liposomes bearing c.11.2101F8 tumors were treated twice a week i.p. injections of 100 mg/kg murine C-IgG or F-IgG plus 2.5 ng murine IL12, and tumor volume was assessed. Depletion of monocytes/macrophages was confirmed by flow cytometric analysis, but this manipulation did not affect the efficacy of combination therapy (Fig. 5E). The difference between mock- and clodronate-depleted mice treated with F-IgG plus IL12 was not significantly different. As other groups have indicated that exhausted or inhibited NK cells may have a negative impact on tumor growth control and survival (30, 31), we examined the potential role of myeloid-derived suppressor cells (MDSC) in modulating the NK cell response to F-IgG–coated tumor cells. Notably, overnight coculture of MDSC with human NK cells at a ratio of 0.5:1 significantly inhibited NK cell lysis of F-IgG–coated KB cells and also reduced the NK cell IFNγ secretion in response to this stimulus (Supplementary Fig. S2). These results indicate that immunosuppressive cells within the tumor microenvironment can affect the NK cell response to F-IgG–coated tumor cells.

Enhancement of murine NK cell cytotoxicity

We evaluated the cytotoxic potential of purified murine NK cells against F-IgG–coated L1210F8 tumor cells in a 51Cr-release assay (20). Folate was conjugated to a murine IgG molecule to create a murine F-IgG construct. Freshly isolated murine NK cells were cultured with low-dose recombinant murine (rm) IL15 (0.5 ng/mL; ref. 32), treated with medium alone or medium containing rnmIL12 (10 ng/mL) and plated with L1210F8 target cells. C-IgG or
Inhibition of tumor growth by IL12 plus F-IgG

Based on our in vitro findings, prior preclinical work from our laboratory, and the results of phase I clinical trials, we chose IL12 for further study (16, 33). The ability of the F-IgG conjugate to penetrate into the tumor microenvironment and specifically bind to tumor cells was evaluated. An F-IgG-FITC construct was utilized to assess its intratumoral penetration in a KB xenograft model. A strong fluorescence signal was detected 72 hours later only in F-IgG-FITC–treated mouse tumors (Fig. 6A, top). Flow cytometric analysis confirmed that specific fluorescence was detectable only in FR+ tumors (Fig. 6A, bottom). Even at this early time point, approximately 40% of the FR+ tumor cells were targeted by F-IgG-FITC. AFR+ murine leukemia tumor model was used to determine whether IL12 could enhance the effect of an antitumor F-IgG construct in an immune competent model. Mice bearing s.c. L1210JF tumors were treated twice a week via i.p. injections of murine C-IgG or F-IgG (at 100 mg/kg), with or without murine IL12 (2.5 ng). No significant tumor volume differences were observed at baseline. However, by day 18 of treatment, the average tumor volumes of mice receiving either IL12 (453 ± 83 mm3) or F-IgG alone (223 ± 68 mm3) were significantly smaller than those of the C-IgG–treated mice (615 ± 152 mm3; P < 0.05). Mice that received the combination of IL12 and F-IgG had significantly smaller tumors (84 ± 43 mm3) than those of mice receiving control treatments (P = 0.05; Fig. 5B). Normal tissues from mice treated with F-IgG exhibited no histologic damage (Supplementary Fig. 3). These data demonstrate that the F-IgG conjugate specifically targets tumor cells, while sparing normal tissues in vivo and that IL12 can enhance the effects of F-IgG in a murine leukemia tumor model.

NK cells are required for the antitumor effects of IL12 and F-IgG

Based on our previous work, we hypothesized that NK cells were the key mediators of the tumor response mediated by IL12 plus F-IgG (16). Mice bearing s.c. L1210JF tumors were depleted of NK cells via anti-asialo GM1 antibodies, which resulted in >97% NK cell depletion (data not shown). Mock-depleted mice received injections of an isotype-matched control antibody. Mice were then treated with IL12 plus F-IgG. Tumor growth was significantly inhibited in mock-depleted mice receiving IL12 and F-IgG (P < 0.0001). In contrast, treatment with F-IgG and IL12 had a minimal effect on tumor growth in the absence of NK cells (Fig. 7A). Activated intratumoral NK cells were observed only in the nondepleted treatment group (Fig. 7B). There was a marked inhibition of Ki67 in mice receiving IL12 plus F-IgG, but not in NK cell–depleted mice, as indicated by immunohistochemical staining (Fig. 7C). These data suggest that the antitumor effect of IL12 and F-IgG is dependent on NK cells.

Discussion

We have demonstrated that an F-IgG construct could bind to the FR of tumor cells. Tumor-bound F-IgG triggered the MAPK pathway in NK cells and coating FR-bearing tumor cells with F-IgG led to enhanced NK cell–mediated ADCC and CD69 expression. Pretreatment of NK cells with IL2, IL12, IL15, or IL21 enhanced ADCC beyond that observed with F-IgG alone. The addition of IL12 to the F-IgG stimulus resulted in robust production of IFNγ, MIP-1α, and RANTES by NK cells, significantly exceeding concentrations observed with either stimulus alone. Cytokines produced from the coculture of NK cells and F-IgG–coated tumor cells treated with IL12 mediated significant T-cell chemotaxis. The conjugate bound to FR+ tumor cells in vivo, but normal tissues showed no obvious adverse effects. Administration of IL12 in combination with F-IgG elicited an antitumor response in a murine tumor model when compared with mice receiving IL12, F-IgG, or C-IgG alone. The antitumor effects of the combination therapy were dependent on NK cells and associated with reduced tumor cell proliferation. These results support the utility of administering cytokines with the F-IgG conjugate as a possible cancer therapeutic.

In murine models, the administration of high doses of folate is linked with tumor progression (34). Although it is not known whether FR expression is needed by malignant cells, folate acid supplementation increases tumor cell growth and decreases patient survival in the context of FR+ tumors (35). Conversely, a blockade of folate uptake decreases tumor cell proliferation, migration, invasion, and tumor burden, implicating folate in the survival, growth, and injury repair of malignant cells (34, 36–38). The reason for FR overexpression in tumor versus normal tissues remains elusive. It is possible that the FR might somehow confer a growth advantage by modulating folate uptake from serum, but direct evidence is lacking (39).

The present folate–IgG construct had binding and intracellular distribution characteristics in FR+ cells that were similar to those of earlier radiolabeled folate-conjugated immunoglobulin constructs (40). Binding assays from studies conducted by Henriksen and colleagues (40) estimated the binding affinity of a folate-conjugated immunoglobulin to be within the Kd range of 10−9 to 10−10 mol/L, which is comparable with the reported high-affinity binding of FRα for folic acid (Kd ∼ 10−7 mol/L; ref. 34). Biodistribution experiments using 125I- and 211At–labeled folate–immunoglobulin conjugates in Balb/C mice indicate that folate conjugation to the antibody has a minor effect on the distribution of the conjugated antibody (40). Therefore, it is reasonable to conclude that the pharmacokinetic parameters of the present F-IgG would be similar to that of other therapeutic IgG-type monoclonal antibodies, including a relatively long half-life (days), low clearance (~0.5 L/d), and a small volume of distribution (approximate to the plasma volume; refs. 41, 42). The results from the present studies indicate that FR binding of F-IgG was evident as early as 30 minutes after treatment and retained on the cell surface for up to 24 hours in vitro. Moreover, in vivo, F-IgG was present in the tumor up to 72 hours after injection.

The FR has been previously utilized for the investigational delivery of cytotoxic drugs, radiopharmaceuticals, nanoparticles, liposomes, and immunotherapy (2, 13). Lu and colleagues (43) showed that folate acid can carry a conjugated hapten to the tumor cell surface (the hapten forming a bridge between the cell surface FR and antibodies to it) and elicit primarily a T cell– and NK cell–based immune response to the tumor. This led to the generation and accumulation of autologous antibodies to haptenons on the tumor surface, stimulating Fc receptor–bearing immune cells to mount an antitumor response against the opsonized tumor cells.
Figure 4.
Human NK cells activated in response to F-IgG-coated KB cells and cytokines secrete IFNγ, MIP-1α, and RANTES and become motile. A, NK cells were cocultured with F-IgG–coated KB tumor (FR-positive) cells in the presence of IL2, IL12, IL15, IL18, or IL21. Control conditions consisted of media only or C-IgG–coated tumor cells in the presence of the cytokines. Culture supernatants were harvested after 48 hours and analyzed for A, IFNγ; B, MIP-1α; and C, RANTES by ELISA. Combinatorial treatment resulted in significant cytokine/chemokine production ($P < 0.0001$). D, T-cell chemotaxis was assessed with coculture supernatants. MIG treatment of media served as a positive control for T-cell migration. *, statistically significant enhancement as compared with all other treatment groups except MIG ($P < 0.01$). E, activated NK cells were assessed via flow cytometric analysis for CD56 and CD69. Results are representative of data derived from $n \geq 2$ donors.
Figure 5. F-IgG plus IL12 enhances monocyte effector functions in vitro but not in vivo. A, the lytic activity of freshly isolated normal monocytes stimulated with IL12 (10 ng/mL) in the presence of C-IgG- or F-IgG-coated KB tumor cells was determined in a standard 18-hour ADCC assay (combinatorial treatment was significantly higher than C-IgG or F-IgG at all E:T ratios with a $P < 0.001$). Normal monocytes were cultured with B, plate-bound F-IgG- or C, F-IgG-coated FR+ KB tumor cells, and culture supernatants were harvested after 48 hours and then analyzed for TNFα and D, IL12 by ELISA (*, $P < 0.04$). Results shown are representative of $n \geq 3$ donors. E, mice depleted of monocytes/macrophages bearing s.c. L1210JF tumors were treated once every 4 days with murine C-IgG (100 mg/kg) or F-IgG (100 mg/kg) plus murine IL12 (2.5 ng).
Figure 6. F-IgG binds to KB cell in vivo, and IL12 enhances its antitumor effects in a murine model. A, mice bearing KB tumors were treated with 100 mg/kg C-IgG-FITC or F-IgG-FITC and sacrificed after 72 hours. Tumors were harvested and fixed/frozen or dissociated. FITC content (green) was assessed in fixed/frozen tissues via confocal microscopy (top). Nuclei are stained blue with DAPI (×200 images). FR status and FITC content were then measured via flow cytometric analysis (bottom). Dual positive FR⁺/FITC⁺ cells indicate specific binding of F-IgG to FR⁺ tumor cells. B, mice bearing s.c. L1210FJ tumors were treated once every 4 days with 100 mg/kg murine C-IgG, 100 mg/kg murine F-IgG, 2.5 ng murine IL12, C-IgG plus IL12 or F-IgG plus IL12. Data are representative of three independent experiments. No significant differences in tumor volume were found among the five groups at baseline. However, 18 days after treatment, the average tumor volumes of mice receiving either IL12 or F-IgG alone were smaller than those of the C-IgG-treated mice (\(^*\), \(P \leq 0.05\)).
Thompson and colleagues (44) devised a method to specifically target T cells to FR-overexpressing tumors utilizing a light-activated folate–anti-CD3 construct. Here, the folate portion of the construct binds to FR+ cancer cells, while the anti-CD3 portion binds T cells. Specific tumor targeting and T-cell binding was observed in a murine model (44). In contrast, the F-IgG construct described here can localize NK cells to tumor cells while simultaneously stimulating FcR-mediated effector functions. Results from these studies support the development of FR-directed therapeutic immunonconjugates and suggest that coadministering cytokines would augment the activity of these reagents. The ability of F-IgG to mediate lysis of target cells derived from both leukemias and solid tumors speaks to the broad applicability of this therapeutic approach.

F-IgG–mediated ADCC was investigated in this study due to the importance of ADCC in the antitumor mechanisms of monoclonal antibody therapy (13, 45, 46). A study in patients receiving trastuzumab for HER2/neu metastatic breast cancers demonstrated that ADCC plays a significant role in the antitumor response (46). Park and colleagues (45) also demonstrated in vivo that the therapeutic effects of anti-HER2/neu antibody occur primarily through ADCC, lending further support to the theory that ADCC is an important mechanism by which antitumor monoclonal antibodies function (10, 45). We have previously shown that the dual engagement of NK cell CD16 and cytokine receptors by cytokines, such as IL2, IL12, or IL21, results in synergistic ADCC and IFNγ production (12, 16, 19, 20, 33). We observed robust and synergistic increases in ADCC and the levels of IFNγ, MIP-1α, and RANTES produced by NK cells when they were exposed to the combination of IL12 and F-IgG-coated tumor cells, as compared with either stimulus alone. Supernatants from cultures of F-IgG and IL12-activated NK cells induced T-cell chemotaxis, highlighting the antitumor microenvironment provided by NK cell costimulation. Administration of IL12 in combination with F-IgG elicited a statistically significant antitumor response that was NK cell–dependent in a murine tumor model. Thus, the coadministration of IL12 and the F-IgG construct could be expected to promote an NK-dependent antitumor response in the clinical setting.

Dual stimulation with IL12 and F-IgG stimulates NK cells to secrete MIP-1α and RANTES, factors that exert a strong chemotactic effect upon monocytes (47). Therefore, NK cell infiltration of F-IgG-treated tumor deposits might be followed by a wave of monocyte migration, especially in the presence of IL12, which further augments NK cell chemokine production. Conversely, monocyte-derived TNFα is a well-known activator of NK cell cytolytic activity (48) and could potentially sensitize tumor targets to NK cell cytolysis and apoptotic stimuli. The concurrent activation of NK cell and monocyte effector functions by IL12 and F-IgG has the potential to induce a synergistic antitumor immune response that relies upon the preprogrammed interdependence of these two innate immune cell populations.

Therapeutically, F-IgG may confer several advantages over other constructs. Studies of F-IgG localization in vivo demonstrate that the conjugate penetrates into the tumor microenvironment and specifically targets tumor cells. About 40% of tumor cells are observed at later time points, with higher doses of F-IgG or that lower F-IgG binding was not detectable by flow cytometry.

The results of the present study suggest that the F-IgG construct is able to promote a direct interaction of FcR-bearing cells with FR-expressing cancer cells, rather than relying on the induction of an Ab response.
Specific engagement of tumor cell surface receptors, while sparing normal tissue, also results in a favorable toxicity profile, as demonstrated in this study. Being fully humanized, hypersensitivity reactions to F-IgG are unlikely to occur (49).

We have shown that F-IgG complexes can be utilized to specifically enhance immune responses by NK cells and monocytes against FR+ malignancies. Treatment of NK cells and monocytes with immune-stimulatory cytokines synergistically augmented their ability to lyse FR-targeted tumor cells, in vitro. In vivo, the coadministration of IL12 and F-IgG resulted in statistically significant tumor reduction, an effect that was dependent on the NK cell compartment. Thus, F-IgG and IL12 can selectively target NK cells to cancer cells by means of the FR.

Disclosure of Potential Conflicts of Interest

Y. Lu is a senior scientist at Merck & Co. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.C. Jaime-Ramirez, E. McMichael, S. Kondadasula, C.C. Skinner, B.L. Mundy-Bosse, E. Luedde, N.B. Jones, A. Mani, K.M. La Perle, Y. Lu, J.P. Butchar, S. Tridandapani


Writing, review, and/or revision of the manuscript: A.C. Jaime-Ramirez, E.L. McMichael, S. Kondadasula, B.L. Mundy-Bosse, A. Mani, Y. Lu, X. Zhang, M. Poi, M. Phelps, J.C. Byrd, R.J. Lee, M.A. Caligiuri, W.E. Carson III

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.C. Jaime-Ramirez, H. Li, J. Li, S. Elavazhagan, Y. Lu, M. Davis, J.C. Byrd

Study supervision: A.C. Jaime-Ramirez, J.P. Butchar, R.J. Lee

Other (helped with paper editing, mouse colony maintenance, and animal experiments): V. Karpa

Other (provided surgical pathology expertise by interpreting HE stained and immunohistochemically stained glass slides): A.C. Schmitt

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