Inhibitory Anti-FLT3 Antibodies Are Capable of Mediating Antibody-Dependent Cell-Mediated Cytotoxicity and Reducing Engraftment of Acute Myelogenous Leukemia Blasts in Nonobese Diabetic/Severe Combined Immunodeficient Mice

Obdulio Piloto,1 Mark Levis,1 David Huso,2 Yiwen Li,3 Hongli Li,3 Mei-Nai Wang,3 Rajiv Bassi,3 Paul Balderes,3 Dale L. Ludwig,3 Larry Witte,3 Zhenping Zhu,3 Daniel J. Hicklin,1 and Donald Small1

1Department of Oncology and 1Comparative Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland and 3ImClone Systems Incorporated, New York, New York

Abstract

Aberrant FLT3 expression and/or mutation plays a significant role in leukemogenesis. This has prompted the development of selective small molecule tyrosine kinase inhibitors against FLT3. However, like most tyrosine kinase inhibitors, those against FLT3 are not completely specific and at the doses required to completely inhibit target, significant toxicities may occur. In addition, tyrosine kinase inhibitors for other kinases have been shown to select for cells that become resistant. To overcome some of these limitations we developed two fully human phage display monoclonal antibodies against FLT3 (IMC-EB10 and IMC-NC7). These antibodies inhibited ligand-mediated activation of wild-type FLT3 and constitutively activated mutant FLT3 and in most cell types affected downstream STAT5, AKT, and mitogen-activated protein kinase activation. In addition to interfering with FLT3 signaling, IMC-EB10 and, to a significantly lesser extent, IMC-NC7 initiated antibody-dependent cell-mediated cytotoxicity on FLT3-expressing cells. When IMC-EB10 was used in vivo to treat nonobese diabetic/severe combined immunodeficient mice given injections of primary FLT3/ITD acute myelogenous leukemia samples or myeloid cell lines with FLT3 expression, it significantly decreased engraftment of leukemic cells and increased survival, respectively. In contrast, IMC-EB10 treatment did not reduce engraftment of normal human CD34+ cord blood cells nor did it show any significant inhibition of normal murine hematopoiesis. Thus, these types of antibodies have the potential to be safe and effective new therapeutic agents for acute myelogenous leukemia and possibly other FLT3-expressing malignancies. (Cancer Res 2005; 65(4): 1514-22)

Introduction

FLT3 is a member of the class III receptor tyrosine kinase family, structurally related to the KIT, FMS and platelet-derived growth factor receptors (1–3). This class of receptor is characterized by five extracellular immunoglobulin-like domains that bind ligand, a single transmembrane domain, and an intracellular kinase domain interrupted by a kinase insert. Ligand binding promotes receptor dimerization and activation of the kinase domain, resulting in autotransphosphorylation of specific tyrosines (4). Activated FLT3 can then phosphorylate target proteins and also serve as a docking site for a variety of proteins with SH2 domains involved in intracellular signaling cascades (5–8). The FLT3 receptor is normally involved in differentiation, proliferation, and survival of multipotent hematopoietic stem cells and lymphoid and dendritic progenitor cells (9). Abnormal expression/activation of FLT3 seems to play various roles in the development of leukemia, particularly acute myelogenous leukemia (AML) and infant acute lymphoid leukemia (reviewed in refs 10–13). FLT3 is often activated by mutations including internal tandem duplications (ITD) in the juxtamembrane domain or point mutations in the kinase domain (14–16). AML patients with FLT3-ITD mutations have a poor prognosis (17–20). In addition, overexpression of wild-type FLT3 with FLT3 ligand also leads to constitutive activation of the FLT3 kinase domain (21–23). The frequency of FLT3 mutations and the poor prognosis associated with them has spurred the development of selective FLT3 tyrosine kinase inhibitors. A number of these inhibitors are able to reduce FLT3 activation in vitro and in vivo, with several inhibitors now in phase I/II clinical trials (24–29). However, like all small molecule inhibitors studied to date, those against FLT3 are selective but not specific and also inhibit various other kinases, especially as their concentration is increased. This decreases the likelihood of ever achieving close to 100% inhibition as the concentrations required would affect enough other kinases to likely result in significant toxicity. Furthermore, as seen with Gleevec, prolonged treatment with small molecule inhibitors can lead to the development of drug resistance, often based on the selection of mutants that no longer bind the drug or through gene amplification (30). Upon continuous drug exposure, similar resistance to FLT3 inhibitors may develop. For these reasons, we decided to explore the possibility of inhibiting FLT3 signaling through alternative methods.

Monoclonal antibodies (mAb) that are specific have been generated against several targets in cancer and are currently in clinical use (31). A specific antibody able to block FLT3 signaling might overcome some of the potential limitations of FLT3 tyrosine kinase inhibitors. Thus, antibody-expressing phage were initially selected for their ability to bind FLT3, and then screened in an ELISA assay to identify antibody fragments capable of blocking FLT3 ligand binding to FLT3. These efforts have resulted in the generation of two fully human mAbs, IMC-EB10 (EB10) and IMC-NC7 (NC7). The ability of IMC-EB10 and IMC-NC7 to reduce FLT3

Note: D. Small is the Douglas Kroll Research Foundation Translational Researcher of the Leukemia and Lymphoma Society and the Kyle Haydock Professor of Oncology at Johns Hopkins.

Requests for reprints: Obdulio Piloto, Johns Hopkins University School of Medicine, 1650 Orleans Street, CRB 276, Baltimore, MD 21231. Phone: 410-955-1554; Fax: 410-955-8897; E-mail: opiloto1@jhmi.edu.

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activation mediated either through activation of mutations or FLT3 ligand stimulation of FLT3 was examined. Inhibiting FLT3 signaling has the potential of limiting growth and inducing apoptosis in FLT3-dependent cells. In addition, antibodies also have the potential to eliminate FLT3-expressing cells through antibody-dependent cell-mediated cytotoxicity. This might expand the use of these antibodies beyond mutant FLT3-expressing patients with AML to include most other cases of AML as well as B-lineage acute lymphoid leukemia, and subsets of T-acute lymphoid leukemia, chronic myelogenous leukemia blast crisis, and chronic lymphocytic leukemia expressing wild-type FLT3. These FLT3 ligand-blocking mAbs also have the potential to specifically disrupt FLT3 signaling without inducing many of the toxicities that can result from small molecule inhibitors. For these reasons, anti-FLT3 mAbs might serve as an effective new therapeutic for the treatment of leukemia.

**Materials and Methods**

**Reagents.** Mouse monoclonal antiphosphotyrosine antibody (4G10) and recombinant protein A-agarose were purchased from Upstate Biotechnol-
ogy, Inc. (Lake Placid, NY); Rabbit anti-PEG-ST5, anti-PEG-AKT, anti-AKT, anti-
P-nitrobenzylated activated protein kinase (MAPK) p44/p42 and anti-MAPK p44/p2 antibodies were obtained from Cell Signaling Technologies, Inc. (Beverly, MA). Rabbit anti-human FLT3 and anti-PEGST5 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Purified human IgG purchased from Jackson Immunoresearch Laboratories (West Grove, PA) and humanized anti-epidermal growth factor receptor antibody, IMC-C225 (Cetuximab; ImClone Systems, New York, NY), were used as controls. Horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection system were purchased from Amersham (Arlington Heights, IL). Recombinant human FLT3 ligand was purchased from PeproTech, Inc. (Rocky Hill, NJ).

**IMC-E10 and IMC-NC7 mAb.** IMC-E10 and IMC-NC7 mAbs were engineered and binding analysis done as previously described (32). Briefly, Fab fragments from a phage display library were screened for their ability to bind FLT3 and candidate genes used to engineer human IgG. These antibodies were further screened for their ability to bind FLT3 and block ligand binding to FLT3 in an ELISA assay. The ability of these two antibodies to bind cell surface wild-type FLT3 and FLT3-ITD, and the ability of FLT3 ligand to compete for binding, was determined by flow cytometric analysis. The binding kinetics of IMC-E10 and IMC-NC7 mAb to FLT3 was measured using a BIACore 2000 biosensor (Pharmacia Biosensor, Uppsala, Sweden).

**Cell Lines.** Cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Rockville, MD), supplemented with 10% heat-inactivated fetal bovine serum (Gimini Bio-Products, Woodland, CA) and penicillin/streptomycin (Life Technologies, Inc.) at 37°C with 5% CO2, Ba/F3 cells transfected with human FLT3, FLT3-ITD, or FLT3-PM were grown under selective conditions of 50 ng/mL IL-3 (Life Technologies, Inc.; ref. 33). The 32D cell line transfected with human FLT3-ITD was grown in the presence of 2 μg/mL puromycin (34).

**Human Samples.** Human samples from patients with AML and normal donors were obtained under an institutional review board-approved protocol at the Sidney Kimmel Cancer Center at Johns Hopkins Hospital. Leukemic blasts were separated by Ficoll-Hypaque (Amersham, Piscataway, NJ) density gradient centrifugation as previously described (35). CD34+ cells were isolated from human cord blood using the Direct CD34 Progenitor Cell isolation Kit (Miltenyi Biotec Inc., Auburn, CA).

**Western Blot Analysis.** Cells were grown overnight in either 0% fetal bovine serum or 10% heat-inactivated fetal bovine serum and treated with control human polyclonal IgG, IMC-E10, or IMC-NC7 mAbs in fresh or conditioned media for 1 to 4 hours at 37°C with 5% CO2. After antibody treatment, cells were washed with cold PBS and lysed for 30 minutes at 4°C in NP40 lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 100 mmol/L NaF, 10% glycerol, 1% NP40, and 10 mmol/L EDTA] containing protease and phosphatase inhibitors (2 mmol/L sodium orthovanadate, 500 μg/mL antipain, 5 μg/mL aprotinin, 1 μg/mL leupeptin, and 10 μg/mL phenylmethylsulfonyl fluoride; Sigma, St. Louis, MO). Lysates (500 μg) were incubated with rabbit anti-human FLT3 antibody overnight followed by incubation with protein A-agarose at 4°C for 2 hours. Immunoprecipitates were washed once with ice-cold TBS-T [10 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 0.1% Tween 20], twice with ice-cold TBS [10 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl], and resuspended in SDS sample buffer, boiled for 5 minutes and separated by 8% SDS-PAGE. Nonimmunoprecipitated total protein lysates were separated by 10% SDS-PAGE. Gels were blotted onto polyvinylidene fluoride microporous membrane (Millipore, Bedford, MA) and probed with the indicated antibody. Antibody binding was detected by incubation with a horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence detection.

**In vitro Antibody-Dependent Cell-Mediated Cytotoxicity Assay.** Antibody-dependent cell-mediated cytotoxicity assays were done by the standard 51Cr release assay. An enrichment of human natural killer (NK) cells was obtained from normal donor blood (RosettaSep NK Cell Enrichment Cocktail; StemCell Technologies, Inc., Vancouver, British Columbia, Canada, or NK Cell Isolation Kit II, Miltenyi Biotec). Target cells (2 × 106) were labeled with 200 μCi 51Cr for 2 hours and then washed. Increasing numbers of NK cells (0 to 400,000 cells), which had been previously incubated with 10 μg/mL (67 mmol/L) control IgG, IMC-C225, IMC-E10, or IMC-NC7 mAb for 45 minutes, were incubated with 4,000 51Cr-labeled target cells, in triplicate in V-bottomed 96-well plates for 6 hours at 37°C, or with 5% SDS to measure total lysis. Supernatant was collected and released 51Cr counted on a gamma counter (1470 Wallac WIZARD, PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). Percent lysis was calculated as 100 × (sample release – spontaneous release)/(total release – spontaneous release).

**In vivo Models of Human Leukemia: Engraftment and Survival.** Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice and nude mice (6-7 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Groups of 10 mice were sublethally irradiated (300 cGy) and given injections of 5 × 10⁶ EOL-1 cells, 5 × 10⁵ Ba/F3-ITD cells, 0.5 × 10⁷ MOLM14 cells, 0.5 × 10⁶ AML blasts expressing FLT3-ITD, or 0.1 × 10⁶ CD34+ human cord blood cells in 500 μL PBS by tail vein injection. Starting the day after cells were injected, mice were treated with i.p. injections thrice per week of 400 μg IMC-E10 or 100, 500, and 1,000 μg IMC-NC7 in 200 μL PBS, 400 μg IMC-C225, or 500 μg of human IgG were used as control treatments. All animal procedures were conducted in conformity with institutional guidelines.

**Flow Cytometry.** Cells were washed in cold IFA buffer (0.01 mol/L HEPES, 0.15 mol/L NaCl, 1% NaN₃, 4% fetal bovine serum, pH 7.4) and stained with human allophycocyanin-conjugated CD13, CD34, FITC-conjugated CD33, phycoerythrin-conjugated CD135, CD45, and murine PerCP-Cy5.5-conjugated CD45 (BD-Bioscience, Palo Alto, CA) for 30 minutes at 4°C in the dark. Each sample was also stained with appropriate isotype controls. Stained cells were washed with cold IFA buffer and incubated with red blood cell lysis buffer (0.155 mol/L NH₄Cl, 0.01 mol/L KCl, 0.1 mmol/L EDTA) for 5 minutes at room temperature and then washed again with cold IFA buffer. Cells were analyzed using a BD-FACS caliber flow cytometer (BD Bioscience, Inc.) and CellQuest software.

**Histology.** Tissue samples were fixed for >24 hours in 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ), embedded in paraffin, and sectioned at 5 μm onto charged slides. Samples containing bone were decalcified with formic acid before sectioning. HE staining was done on an automated stainer.

**Results**

**Binding and Blocking Characterization of IMC-NC7.** IMC-NC7 is a human anti-FLT3 mAb (IgG1, n) that was identified from a naïve human antibody phage display library for its ability to firmly bind FLT3-Fc in an ELISA (Fig. 1A). To assay for specificity, the binding assay was done with a control fibroblast growth factor-Fc...
fusion protein with no antibody binding observed (data not shown). The BioCore analysis of IMC-NC7 binding to FLT3 shows that $K_D = 450$ pmol/L, with association and dissociation constants of $1.24 \times 10^5$ mol L$^{-1}$ s$^{-1}$ and $5.6 \times 10^{-5}$ s$^{-1}$, respectively. The affinity of IMC-NC7 for FLT3 is similar to that of FLT3 ligand (200-500 pmol/L; ref. 36). The epitope bound by IMC-NC7 seems to overlap with that of FLT3 ligand and a competition ELISA shows that IMC-NC7 can block FLT3 ligand binding to FLT3 (Fig. 1A).

Fluorescence-activated cell-sorting analysis reveals that IMC-NC7 binds to both wild-type and mutant FLT3 on human eosinophilic leukemia cells (EOL-1) and Ba/F3-ITD cells, respectively (Fig. 1C). IMC-NC7 (10 µg/mL, 67 nmol/L) binding to Ba/F3-ITD cells can be reduced by addition of FLT3 ligand (100 ng/mL), further supporting the notion that IMC-NC7 and FLT3 ligand bind to FLT3 at an overlapping site (Fig. 1D).

IMC-EB10 was previously characterized with an association and dissociation constant for FLT3 of $3.52 \times 10^5$ mol L$^{-1}$ s$^{-1}$ and $5.55 \times 10^{-5}$ s$^{-1}$, respectively ($K_D = 158$ pmol/L; ref 32). IMC-EB10 inhibited wild-type and mutant FLT3 in vitro and IMC-EB10 treatment of mice given injections of EOL-1 and Ba/F3-ITD cells prolonged survival.

**IMC-NC7 mAb Blocks FLT3 Ligand–Mediated Wild-type and FLT3 Ligand–Independent Mutant FLT3 Activation along with Subsequent Activation of Downstream Pathways.** Activated FLT3 signaling clearly plays a role in AML and therefore an anti-FLT3 mAb may provide a therapeutic effect by interfering with FLT3 signaling. One of the ways FLT3 is activated is through FLT3 ligand binding with subsequent receptor dimerization and autotransphosphorylation (37). IMC-EB10 and IMC-NC7 were originally selected for their ability to bind FLT3 and block FLT3 ligand binding in an ELISA. IMC-NC7 blocks FLT3 ligand–mediated FLT3 activation in the murine pro-B cell line, Ba/F3, transfected with wild-type FLT3 (Ba/F3-FLT3; Fig 2A, lanes 4-6 versus 2 and 3). The inhibition of FLT3 activation also results in a reduction in downstream AKT and MAPK phosphorylation (Fig. 2A, lanes 4-6 versus 2 and 3). In contrast, STAT5 phosphorylation is not affected by IMC-NC7. This may be a reflection of the limited role the STAT5 pathway plays in wild-type FLT3 signaling, in contrast to its role in mutant FLT3 signaling (8, 24, 38, 39). Incubation of a control human IgG polyclonal antibody with Ba/F3-FLT3 cells does not affect FLT3, STAT5, AKT, or MAPK activation (Fig. 2A, lane 3).

FLT3 can also be activated without FLT3 ligand by the presence of an ITD mutation (40, 41). Thus, one might predict that the anti-FLT3 antibody would have very little effect on ITD mutant FLT3 activation. To examine this possibility, IMC-NC7 was used to treat murine myeloid 32D cells transfected with FLT3-ITD (Fig. 2B). FLT3-ITD 32D cells show constitutive phosphorylation of FLT3 and downstream signaling proteins including STAT5, AKT, and MAPK in the absence of added FLT3 ligand (Fig. 2B, lane 1) and this activation is unaffected by incubation with control IgG (Fig. 2B, lane 2). IMC-NC7 caused a dose-dependent inhibition of FLT3-ITD phosphorylation as well as phosphorylation of STAT5, AKT, and MAPK (Fig. 2B, lanes 1 and 2 versus 3-5).

The other common class of FLT3 activating mutations consists of point mutants in the kinase domain (14, 16). The most frequently occurring mutation results in the replacement of aspartic acid 835 with tyrosine (D835Y; ref 14). FLT3(D835Y) and downstream signaling proteins including STAT5, AKT, and MAPK are constitutively phosphorylated in Ba/F3-D835Y even in the presence of control IgG (Fig. 2C, lane 1). Phosphorylation of FLT3 (D835Y) is partially inhibited by IMC-NC7 (Fig. 2C, lanes 2-4), with significant effects in downstream signaling.

**IMC-NC7 Is More Effective than IMC-EB10 at Blocking FLT3 Ligand–Mediated FLT3 Activation.** IMC-EB10, which was previously characterized, is also able to inhibit wild-type and mutant FLT3 signaling as well as downstream pathways (32). To compare the ability of IMC-EB10 and IMC-NC7 to block FLT3

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**Figure 1.** IMC-NC7 binds wild-type and mutant FLT3 and competes with FLT3 ligand for FLT3 binding. A, IMC-NC7 binding to FLT3 was measured by ELISA, using plates coated with FLT3-Fc fusion protein and detected with horseradish peroxidase–conjugated anti-human mAb antibody. B, IMC-NC7 competition of FLT3 ligand binding to FLT3 was measured by a competition ELISA by which biotinylated FLT3-Fc fusion protein, preincubated with IMC-NC7, was incubated with wells coated with FLT3 ligand. FLT3 bound to FLT3 ligand was detected by streptavidin–horseradish peroxidase conjugate. C, IMC-NC7 binding to cell surface wild-type FLT3 on EOL-1 cells and mutant FLT3 on Ba/F3-ITD cells was determined by fluorescence-activated cell-sorting analysis using a phycoerythrin-conjugated anti-human secondary antibody. Ctrl, control. D, FLT3 ligand (FL) competition of IMC-NC7 binding to cell surface FLT3 on Ba/F3-ITD cells was determined by fluorescence-activated cell-sorting analysis using a phycoerythrin-conjugated anti-human secondary antibody.
lignand–mediated activation of wild-type FLT3 we used EM3 cells. EM3 is a chronic myeloid leukemia-derived cell expressing wild-type FLT3 and its activation is completely dependent on addition of FLT3 ligand. Addition of 1 μg/ml (6.7 nmol/L) IMC-EB10 or IMC-NC7 blocks FLT3 ligand–mediated FLT3 phosphorylation partially and completely, respectively (Fig. 3, lanes 3 and 7 versus 2). Addition of increasing amounts of FLT3 ligand outcompetes IMC-EB10 but not IMC-NC7, showing that IMC-NC7 is more potent than IMC-EB10 at blocking FLT3 ligand activation of FLT3 (Fig. 3, lanes 7-10 versus 3-6).

**IMC-EB10 and IMC-NC7 Interfere with FLT3 Signaling in Primary AML Blasts.** The ability of IMC-EB10 and IMC-NC7 to inhibit FLT3 signaling in three wild-type and two mutant FLT3-expressing primary AML blasts was also tested. Upon thawing and overnight incubation, AML blast samples expressing wild-type FLT3 (Fig. 4A, lane 1) or FLT3-ITD (Fig. 4B, lane 1) both show constitutive activation of FLT3. Phosphorylation of FLT3 is not affected by incubation with control IgG (Fig. 4A and B, lane 2). Addition of either antibody strongly inhibited FLT3 phosphorylation of wild-type FLT3 (Fig. 4A, lanes 3 and 4). There seemed to be only slight, if any, inhibition of the downstream signaling pathways involving STAT5, AKT, and MAPK in these primary cells. This may reflect the lesser role that wild-type FLT3 signaling plays in leukemia cells, with many other signaling pathways able to activate these downstream molecules. Only IMC-NC7 seemed to inhibit FLT3-ITD phosphorylation in primary cells and the inhibition was only partial (Fig. 4B, lane 4). Little change was seen in the phosphorylation of STAT5, AKT, and MAPK.

**IMC-EB10 mAbs, and IMC-NC7 to a Significantly Lesser Extent, Mediate Antibody-Dependent Cell-Mediated Cytotoxicity of FLT3-Expressing Cells.** Despite blocking FLT3 signaling in most cell lines, IMC-EB10 and IMC-NC7 treatment alone did not significantly reduce proliferation or induce apoptosis, as measured in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Annexin V binding assays, respectively (data not shown). However, antibodies against FLT3 could still prove efficacious if they are able to elicit antibody-dependent cell-mediated cytotoxicity, whether or not FLT3 and downstream signaling is inhibited or is critical for cell survival. To examine this possibility, parental Ba/F3 cells and Ba/F3 cells transduced with wild-type FLT3 or FLT3-ITD were labeled with 51Cr for 2 hours and then incubated with the antibodies and increasing numbers of NK cells for 6 hours. IMC-EB10 mediated antibody-dependent cell-mediated cytotoxicity against Ba/F3-FLT3 and Ba/F3-ITD cells as shown by 51Cr release (Fig. 5A and B). Despite its inferior ability to inhibit FLT3 signaling, IMC-EB10 was more effective than IMC-NC7 at mediating antibody-dependent cell-mediated cytotoxicity. Control IgG or IMC-C25 resulted in minimal cell lysis even at the highest level of NK cells added. Neither control IgG, IMC-C25, IMC-EB10, or IMC-NC7 stimulated cell lysis in the absence of added NK cells. When NK cells were replaced with peripheral blood mononuclear cells depleted of NK cells, neither IMC-EB10 or IMC-NC7 mediated significant cell lysis against Ba/F3-FLT3 or Ba/F3-ITD cells (data not shown). In addition, treatment with IMC-EB10 and IMC-NC7 did not result in cell lysis of parental Ba/F3 cells not expressing FLT3, even at the highest level of added NK cells (data not shown). Taken together, this shows that IMC-EB10 and, to a significantly lesser extent IMC-NC7, can selectively target wild-type FLT3- and FLT3-ITD-expressing cells for lysis by NK cells.

**IMC-EB10 Significantly Prolongs Survival of NOD/SCID Mice Injected With MOLM14 Cells.** Correlating with the decreased ability of IMC-NC7 to induce antibody-dependent cell-mediated cytotoxicity, no increased survival was seen in IMC-NC7-treated NOD/SCID or nude mice given injections of EOL-1 or Ba/F3-ITD cells, respectively. Thus, only IMC-EB10 was used for further in vivo experiments. The in vivo efficacy of IMC-EB10 was determined using NOD/SCID mice given injections of 0.5 × 10⁶ MOLM14 cells. MOLM14 is a human AML cell line expressing both wild-type and mutant (ITD) FLT3 and therefore better approximates the typically aggressive human FLT3 mutant AML than the murine lymphoid Ba/F3 cell line transfected with FLT3-ITD or EOL-1 cells used in previous in vivo studies (32). Median survival of PBS-treated mice was 36 days (Fig. 6). Treatment of these mice with 400 μg IMC-EB10 thrice per week significantly prolonged survival. Median survival has not yet been reached at >100 days post cell injection.

**IMC-EB10 Reduces Engraftment of Human Primary AML Cells in NOD/SCID Mice without Reducing Engraftment of Normal Human Cord Blood CD34+ Cells.** To assess the ability of the antibodies to reduce leukemia in vivo, we utilized the NOD/SCID model of human leukemia. In this model, sublethally

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**Figure 2.** IMC-NC7 blocks FLT3 ligand–mediated activation of wild-type FLT3 and FLT3 ligand–independent activation of mutant FLT3 along with downstream signaling. Serum-starved (A) Ba/F3-FLT3 cells, (B) 32D/FLT3-ITD, and (C) Ba/F3-FLT3(D835Y) cells (10 × 10⁶ cells/ml) were incubated with PBS, control IgG, or IMC-NC7 for 1 to 2 hours. Ba/F3-FLT3 cells were stimulated with 50 ng/ml FLT3 ligand for 15 minutes. Immunoprecipitates and total protein extracts were resolved by 8% or 10% SDS-PAGE, respectively, and subjected to immunoblot analysis with the indicated phosphospecific antibodies. The same blots were then stripped and reprobed with protein-specific antibodies.
irradiated NOD/SCID mice are given injections of primary human leukemia samples. Previous work has shown that the ability of leukemia cell samples to engraft these mice is variable, with about 50% of patient samples successfully growing in the mice (42, 43). The cells that grow out over a 3- to 4-month period are reflective of leukemic stem cell activity as cell fractions depleted for those cells are unable to engraft mice at any cell dose (44). In addition, the aggressive nature of FLT3-ITD leukemia has been found to correlate with a better ability to engraft NOD/SCID mice (42, 43). Thus, two samples of primary AML blasts expressing mutant FLT3-ITD were used to test the efficacy of EB10 treatment. Representative results from one of the two primary samples is shown in Fig. 7A to F. Because IMC-NC7 failed to prolong survival of mice given injections of leukemic cell lines, IMC-EB10 was used to treat the mice and was given thrice per week via i.p. injections.

Flow cytometric analysis of bone marrow cells from NOD/SCID mice indicate that IMC-EB10 is effective at reducing the level of engraftment of human AML cells expressing FLT3-ITD at both 6 and 14 weeks post cell injection (Fig. 7A and C). Peripheral blood smears obtained from mice at these same time points also reveal fewer circulating blasts in mice treated with IMC-EB10 (Fig. 7B and D). Normal murine hematopoiesis was not affected as no significant differences between the two groups were noted for peripheral blood white blood cell, red blood cell, neutrophil, lymphocyte, monocyte, eosinophil, basophil, hematocrit, hemoglobin, or platelet counts (data not shown).

Grossly, the bone marrow of the PBS-treated mice given AML injections appeared white, whereas that of the IMC-EB10-treated mice appeared a normal red hue. Histological analysis of the bone marrows from the PBS-treated mice showed a marrow in which the normal hematopoietic cells were replaced with uniform sheets of leukemic blasts (Fig. 7F). In contrast, the IMC-EB10-treated mice have a more normal-looking bone marrow, with multiple cell lineages represented (Fig. 7F). No evidence of leukemic cell infiltration into the spleen, liver, or brain of untreated or IMC-EB10-treated mice was observed (data not shown).

To minimize toxicity, therapeutic agents developed against hematologic malignancies should specifically target malignant cells without significantly affecting normal hematopoiesis. In contrast to the observed reduction in engraftment of AML primary samples, 14 weeks of IMC-EB10 treatment did not significantly affect the engraftment of normal human cord blood CD34+ cells (Fig. 7G). Histological analysis of the bone marrows from PBS/
This study presents data on the use of two human anti-FLT3 mAbs (IMC-EB10 and IMC-NC7) that take advantage of FLT3 as a molecular target. They have the potential to overcome at least some of the shortcomings of small molecule inhibitors. Monoclonal antibodies, as therapeutics, have the advantage of being more specific and thus less toxic compared with small molecule inhibitors. IMC-EB10 and IMC-NC7 block FLT3 ligand–mediated activation of FLT3 in cell lines and primary AML cells expressing wild-type FLT3. IMC-NC7 seems to be more potent than IMC-EB10 at blocking this activation. Proteins downstream of wild-type FLT3 signaling, including AKT and MAPK, are also inhibited when FLT3 ligand–stimulated FLT3 cells are incubated with IMC-EB10 or IMC-NC7. The differences in the ability of the two antibodies to block FLT3 ligand activation of FLT3 might be explained by differences in the epitopes bound. This might confer varying abilities to sterically block FLT3 ligand binding to FLT3.

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Because IMC-EB10 and IMC-NC7 were selected both for their ability to bind the extracellular portion of FLT3 and to block FLT3 ligand binding to FLT3, we did not expect IMC-EB10 or IMC-NC7 to necessarily interfere with FLT3 activated by mutations (ITD or point mutations). However, immunoblots showed that IMC-EB10 and IMC-NC7 partially inhibit phosphorylation of mutant FLT3 and, in turn, activation of downstream FLT3 signaling pathways including STAT5, AKT, and MAPK. In contrast, incubation of AML primary samples with IMC-EB10 and IMC-NC7 resulted in little to no inhibition of FLT3-ITD and downstream pathways. Unlike cell lines, AML primary samples could not be serum starved overnight before in vitro studies because of viability issues, which may explain the observed discrepancy.

How these antibodies might interfere with mutant FLT3 activation/signaling is an interesting question. IMC-EB10 and IMC-NC7 may interfere with receptor dimerization or directly induce a conformational change that inhibits FLT3 kinase activity. These antibodies may also interfere with downstream signaling by inducing FLT3 conformational changes that interfere with binding of direct targets of phosphorylation or others that bind through SH2 domains. This idea is supported by the finding that phosphorylation of downstream targets is sometimes inhibited in the mutants out of proportion to the inhibition of FLT3-ITD and FLT3(D835Y) autophosphorylation. Antibody-mediated FLT3 internalization may also explain why downstream pathways are sometimes inhibited out of proportion to mutant FLT3 inhibition. Alternatively, perhaps FLT3 mutants are still at least partially dependent on FLT3 ligand activation. The decrease in phosphorylation would then be due to a block in FLT3 ligand activation of FLT3 mutants. Most or all leukemia cells have recently been shown to coexpress FLT3 ligand (21). Therefore the observed independence of FLT3-ITD and FLT3/kinase domain mutant activation on exogenously added FLT3 ligand could actually be the result of sensitization toward ligand that would be satisfied by FLT3 ligand–mediated autocrine activation of FLT3. This mode of activation could still be inhibited by the anti-FLT3 antibodies.

The data show that IMC-EB10 and IMC-NC7 can inhibit FLT3 phosphorylation in cell lines and primary AML samples, and that this inactivation results in inhibition of signaling proteins downstream of FLT3. Small molecule FLT3 inhibitors (i.e., AG1295, CEP-701, PKC412, MLN518, and SU11248) also inhibit FLT3 activation and induce apoptosis in cells dependent on FLT3 stimulation (24, 25, 27–29). In contrast, incubation of murine and human cell lines with IMC-EB10 or IMC-NC7 does not significantly induce apoptosis or reduce cell proliferation as measured by the MTT and Annexin V binding assays (data not shown). Some reduction in [3H]thymidine incorporation was observed when IMC-EB10 was used to treat Ba/F3-ITD cells and FLT3 ligand–stimulated EOL-1 cells (32). This difference in response to tyrosine kinase inhibitor versus antibodies might be due to the antibodies’...
failure to reach and sustain a high enough level of FLT3 inhibition to pass a critical threshold required to significantly reduce proliferation and induce apoptosis. The difference might also be the result of additional nonspecific effects induced by the small molecule inhibitors, which combines with their inhibition of FLT3 kinase activity to induce a cytotoxic response.

Although unable to induce a cytotoxic response by themselves, IMC-EB10 and, to a significantly lesser extent, IMC-NC7 effectively lyse FLT3-expressing cells in vitro through the antibody-dependent cell-mediated cytotoxicity pathway. This cell lysis required the addition of both NK cells and antibody specifically bound to FLT3. The difference in antibody-dependent cell-mediated cytotoxicity abilities between IMC-EB10 and IMC-NC7 might reflect the differences in how they bind to FLT3. This difference in antibody-dependent cell-mediated cytotoxicity ability may explain why IMC-EB10 but not IMC-NC7 prolongs survival of

Figure 7. IMC-EB10 reduces engraftment of primary human AML FLT3-ITD blasts in NOD/SCID mice without reducing engraftment of human cord blood CD34+ cells. NOD/SCID mice received injections via tail vein of $0.5 \times 10^6$ FLT3-ITD positive primary AML or $0.1 \times 10^6$ human cord blood CD34+ cells were treated with 400 μg IMC-EB10 i.p. thrice per week. PBS or IMC-C225 was injected into the other mice as control treatments. Bone marrow engraftment of human cells was determined by harvesting the femurs and analyzing cells for human CD45 by flow cytometry at (A) 6 and (C) 14 weeks post injection. The presence of blasts in the peripheral blood was determined by morphologic analysis of blood smears at (B) 6 and (D) 14 weeks post leukemic cell infection and reported as a ratio of blasts to neutrophils. AML blasts were visually detected by H&E-stained bone marrow sections of (E) PBS- and (F) IMC-EB10-treated mice. Magnification ×200. Bar, 20 μm. G, bone marrow engraftment of human cord blood CD34+ cells was determined by flow cytometry at 14 weeks post cell injection.

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mice given injections of wild-type and mutant FLT3-expressing leukemic cells because both antibodies can inhibit FLT3 signaling and downstream pathways. Furthermore, the observation that IMC-NC7 can block FLT3 ligand–mediated FLT3 activation better than IMC-EB10 but shows no significant in vivo efficacy emphasizes that the main cytotoxic mechanism used by these antibodies is more likely to be antibody-dependent cell-mediated cytotoxicity as opposed to direct FLT3 inhibition. However, antibody inhibition of the antipapoptotic signal provided by FLT3 signaling may enhance cell lysis through antibody-dependent cell-mediated cytotoxicity. Antibody-mediated internalization and down-modulation of cell-surface FLT3 would also decrease FLT3 signaling.

IMC-EB10 reduces engraftment in NOD/SCID mice of two primary human AML samples expressing FLT3-ITD mutations but does not reduce engraftment of human cord blood CD34+ cells. The lack of cytotoxicity toward normal hematopoietic stem cells may be reflective of the lower expression of FLT3 by these cells. Leukemic cells were decreased in both bone marrow and peripheral blood at both early and late times after injection. Because NOD/SCID mice lack significant hemolytic complement activity, complement fixation is unlikely to explain the observed reduction in leukemic cells (49). As shown in Fig. 4B for one of the two primary AML samples used for the in vivo studies, IMC-EB10 did not significantly inhibit FLT3 signaling in either AML sample. This further supports the idea that the observed reduction in engraftment is probably not due to FLT3 inhibition, and more likely reflects antibody-dependent cell-mediated cytotoxicity activity. NOD/SCID mice have reduced NK cell activity compared with nonimmunodeficient mice, and thus, the effects might be even larger in more immune competent hosts (49).

To conclude, the data show in vitro and in vivo that anti-FLT3 antibodies can interfere with FLT3 signaling, prolong survival, and reduce the engraftment of AML cells expressing FLT3-ITD without significantly reducing engraftment of hematopoietic stem cells. Such antibodies might prove efficacious by both interfering with FLT3 signaling and by mediating cell killing through antibody-dependent cell-mediated cytotoxicity. This latter ability might make the antibodies effective not only in FLT3 mutant leukemias, in which FLT3 signaling seems to be important for the survival of the cells but also in other cases of AML, acute lymphoid leukemia, and other leukemias in which wild-type FLT3 is expressed. These antibodies might also prove to have less toxicity than antibodies directed against CD33 in AML because of decreased suppression of neutrophils. Additional in vivo testing of these antibodies for efficacy in a wide spectrum of leukemias, both as naked antibodies and as cytotoxic-conjugated antibodies, is warranted. Such studies will identify the appropriate patient population and antibody to move into clinical trials.

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References

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Obdulio Piloto, Mark Levis, David Huso, et al.


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