IMC-EB10, an Anti-FLT3 Monoclonal Antibody, Prolongs Survival and Reduces Nonobese Diabetic/Severe Combined Immunodeficient Engraftment of Some Acute Lymphoblastic Leukemia Cell Lines and Primary Leukemic Samples

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Abstract

The class III receptor tyrosine kinase FLT3 is expressed on the blasts of >90% of patients with B-lineage acute lymphoblastic leukemias (ALL). In addition, it is expressed at extremely high levels in ALL patients with mixed lineage leukemia rearrangements or hyperdiploidy and is sometimes mutated in these same patients. In this report, we investigate the effects of treating ALL cell lines and primary samples with human anti-FLT3 monoclonal antibodies (mAb) capable of preventing binding of FLT3 ligand. *In vitro* studies, examining the ability of two anti-FLT3 mAbs (IMC-EB10 and IMC-NC7) to affect FLT3 activation and downstream signaling in ALL cell lines and primary blasts, yielded variable results. FLT3 phosphorylation was consistently inhibited by IMC-NC7 treatment, but in some cell lines, IMC-EB10 actually stimulated FLT3 activation, possibly as a result of antibody-mediated receptor dimerization. Through antibody-dependent, cell-mediated cytotoxicity, such an antibody could still prove efficacious against leukemia cells *in vivo*. In fact, IMC-EB10 treatment significantly prolonged survival and/or reduced engraftment of several ALL cell lines and primary ALL samples in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. This occurred even when IMC-EB10 treatment resulted in FLT3 activation *in vitro*. Moreover, fluorescence-activated cell sorting and PCR analysis of IMC-EB10-treated NOD/SCID mice surviving 150 days post-leukemic cell injection revealed that FLT3 immunotherapy reduced leukemic engraftment below the level of detection in these assays (<0.001%). Furthermore, *in vivo* IMC-EB10 treatment did not select for resistant cells, because cells surviving IMC-EB10 treatment remain sensitive to IMC-EB10 cytotoxicity upon retransplantation. *In vivo* studies involving either partial depletion or activation of natural killer (NK) cells show that most of the cytotoxic effect of IMC-EB10 is mediated through NK cells. Therefore, such an antibody, either naked or conjugated to radioactive isotopes or cytotoxic agents, may prove useful in the therapy of infant ALL as well as childhood and adult ALL patients whose blasts typically express FLT3. (Cancer Res 2006; 66(9): 4843-51)

Note: ImClone Systems, Inc. provided some of the funding for the studies reported here. In addition, several of the authors are employed by ImClone Systems, whose potential product was studied in this report.

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doi:10.1158/0008-5472.CAN-06-0018

Introduction

The class III receptor tyrosine kinase FLT3 is composed of five extracellular immunoglobulin-like domains that bind a ligand, a single transmembrane domain, and an intracellular kinase domain interrupted by a kinase insert (1–3). Ligand binding promotes receptor dimerization and activation of the kinase domain, leading to autotransphosphorylation of specific tyrosines (4). Activated FLT3 mediates signaling by phosphorylating target proteins and by serving as a docking site for SH2 domain containing proteins (5–8).

Normally, the FLT3 receptor is involved in differentiation, proliferation, and survival of multipotent hematopoietic stem cells as well as lymphoid and dendritic progenitor cells (9). Aberrant FLT3 expression/activation has been shown to play various roles in leukemogenesis, particularly in acute myelogenous leukemia (AML) and mixed lineage leukemia (MLL)–rearranged acute lymphoid leukemia (ALL) (reviewed in refs. 10–13). FLT3 may be activated by internal tandem duplication mutations in the juxtamembrane region or by point mutations in the kinase domain (14–16). Alternatively, wild-type (wt) FLT3 can be constitutively phosphorylated through overexpression of FLT3 along with ligand stimulation through an autocrine pathway (17–19). FLT3 has been shown to be expressed by >90% of B-lineage ALL blasts and is expressed at extremely high levels in ALL patients with MLL rearrangements or hyperdiploidy and is sometimes mutated in these same patients (20–22). These characteristics make FLT3 an attractive receptor for targeted therapy; thus, tyrosine kinase inhibitors (TKI) against FLT3 have been developed. Some of these TKIs selective for FLT3 are able to sufficiently decrease FLT3 activation to induce apoptosis *in vitro* and *in vivo* (23–28). Several FLT3 TKIs have been tested in phase I/II clinical trials in patients with AML. TKIs targeting FLT3 are selective but not specific and thereby inhibit various other kinases that lead to some toxicities observed in those trials. In addition, prolonged treatment with TKIs are likely to select for resistance through mutation of FLT3, as was observed in BCR-ABL with Gleevac treatment of chronic myelogenous leukemia (CML; ref. 29).

To overcome some of these limitations, we decided to investigate the targeting of FLT3 by monoclonal antibodies (mAb). A number of mAbs have been shown to successfully target cell surface receptors and interfere with vital signaling pathways (30). Besides inhibiting receptor activation, mAbs have the added ability of recruiting the host's immune system against the targeted cells and thereby killing cells that do not depend on receptor activation for survival. Anti-FLT3 mAbs IMC-EB10 and IMC-NC7 have been shown to inhibit wt and constitutively active mutant FLT3 in AML-derived cell lines and primary AML samples (31, 32). Furthermore,

IMC-EB10 treatment of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice given AML blasts or cell lines resulted in reduced engraftment and/or prolonged survival without affecting engraftment of cord blood $CD34^+$ cells (32). In this report, we examine the efficacy of anti-FLT3 immunotherapy *in vitro* and *in vivo* against ALL-derived cell lines and primary ALL samples and investigate the mechanism employed by IMC-EB10 to induce cytotoxicity *in vivo*.

Materials and Methods

Reagents. Mouse monoclonal anti-phosphotyrosine antibody (4G10) and recombinant protein A-agarose were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit anti-phospho-signal transducers and activators of transcription 5 (anti-phospho-STAT5), anti-phospho-AKT, anti-AKT, anti-phospho-mitogen-activated protein kinase (anti-phospho-MAPK) p44/42, and anti-MAPK p44/42 antibodies were obtained from Cell Signaling Technologies, Inc. (Beverly, MA). Rabbit anti-human FLT3 and anti-STAT5 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Humanized anti-epidermal growth factor receptor (anti-EGFR) antibody IMC-C225 (Cetuximab; ImClone Systems, New York, NY) was used as a control. Asialo GM1 antisera was obtained from Wako Pure Chemical Industries (Osaka, Japan), and polyinosinic-polycytidylic acid [poly (I:C)] was obtained from Sigma (St. Louis, MO). Horseradish peroxidase–conjugated secondary antibody and enhanced chemiluminescence detection system were purchased from Amersham (Arlington Heights, IL).

IMC-EB10 and IMC-NC7 mAbs. IMC-EB10 and IMC-NC7 mAbs were engineered and binding analysis done as previously described (31, 32).

Cell lines. Human cell lines were cultured in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gimini Bio-Products, Woodland, CA) and penicillin/streptomycin (Life Technologies) at 37°C with 5% CO_2.

Human samples. Human samples from pediatric ALL patients 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 (33).

Western blot analysis. Western blots were done as previously described (32). Briefly, cells were treated for 1 hour with 10 μg/mL IMC-C225, IMC-EB10, or IMC-NC7 mAbs in fresh RPMI supplemented with 10% heat-inactivated FBS. Cells were then washed with cold PBS and lysed for 30 minutes at 4°C in NP40 lysis buffer. 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. Protein A-agarose beads were washed with TBS-T and TBS, resuspended in SDS sample buffer, boiled for 7 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 vivo* models of human leukemia: engraftment and survival.** NOD/SCID mice were bred at Johns Hopkins University School of Medicine. Mice were sublethally irradiated (300 cGy) and injected with 0.5×10^6 cells from ALL-derived cell lines or 1×10^6 cells from ALL primary samples in 500 μL PBS by tail vein injection. Mice were given i.p. injections of 400 μg of IMC-C225 or IMC-EB10 at the indicated times. All animal procedures were conducted in conformity with institutional guidelines.

Flow cytometry. Cells were washed in chilled IFA buffer [0.01 mol/L HEPES, 0.15 mol/L NaCl, 1% NaN_3, 4% FBS (pH 7.4)] and stained with human APC-conjugated CD45, FITC-conjugated CD19, PE-conjugated CD135, and/or murine PE-conjugated CD45 or DX5 (BD Biosciences, Inc., 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 RBC lysis buffer (0.155 mol/L NH_4Cl, 0.01 mol/L $KHCO_3$, 0.1 mmol/L EDTA) for 3 minutes at room temperature and then washed again with cold IFA buffer. Cells were analyzed using a BD-FACSCalibur flow cytometer (BD Biosciences) and CellQuest software.

Quantitative real-time PCR. DNA was isolated from murine bone marrow and spleen cells using the QIAamp DNA Mini kit (Qiagen Sciences, Valencia, CA). Isolated DNA was quantified using the QuantiTect SYBR Green PCR Kit (Qiagen Sciences) and an iCycler iQ multicolor real-time PCR system (Bio-Rad, Richmond, CA). PCR cycles were 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Cloned-actin cDNA was used as a standard for quantification using specific sense (5-TGCGTGACATTAAGGAGAAG-3) and antisense (5-GCTCGTAGCTCTTCTCCA-3) primers. Human-specific glyceraldehyde-3-phosphate dehydrogenase (h-GAPDH) primers (5-CAACGAATTTGGCTACAGCA-3 and 5-CCCCTCTTCAAGGGGTCTAC-3) were used to identify human DNA. Primers (5-ACCACAGTCCATGCCATCAC-3 and 5-TCCACCACCCTGTTGCTGTA-3) that amplify both murine and human GAPDH (m-GAPDH/h-GAPDH) DNA were used as loading controls. DNA samples from murine spleen/bone marrow cells added to human SEM-K2 cells at specific cell ratios were used as a standard curve for estimating the percentage of human cells. Gene copy numbers were determined from the threshold amplification cycle numbers using software supplied with the iCycler IQ Thermal Cycler. PCR products were visualized on a 2% agarose gel.

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. H&E staining was done on an automated stainer.

Results

IMC-NC7 inhibits FLT3 activation along with downstream signaling pathways, whereas IMC-EB10 variably activates or inhibits these same pathways in ALL cell lines. FLT3 activation has been shown to play a role in leukemia, and inhibition of FLT3 activation in FLT3-dependent cells using TKIs results in apoptosis (8, 10–13, 23–27). The ability of IMC-EB10 and IMC-NC7 to affect FLT3 signaling was investigated using a number of ALL-derived cell lines expressing differing levels of cell surface FLT3 (Fig. 1*A*). We first treated two ALL-derived cell lines (Karpas45 and NALM6), showing very low levels of FLT3 expression (Fig. 1*A* and *B*). Neither IMC-EB10 nor IMC-NC7 significantly affected the activation status of FLT3 or downstream signaling pathways when compared with IMC-C225 treatment (Fig. 1*B*, *lanes 2* and *3* versus *lane 1, lanes 5* and *6* versus *lane 4*). In contrast, IMC-NC7 treatment of ALL-derived cell lines expressing constitutively active FLT3-D835H (Hb1119) or constitutively active wt FLT3 (SEM-K2, REH, and RS411) resulted in significant suppression of FLT3 phosphorylation and activation of downstream pathways compared with treatment with control antibody, IMC-C225 (Fig. 1*B*, *lane 9* versus *lane 7, lane 12* versus *lane 10, lane 15* versus *lane 13, lane 18* versus *lane 16*). IMC-NC7-mediated inhibition of FLT3 phosphorylation is less dramatic in Hb1119 cells than in SEM-K2, REH, or RS411 cells possibly due to expression of FLT3 ligand–independent, mutant FLT3 on Hb1119 cells (Fig. 1*B*, *lane 9* versus *lanes 12, 15*, and *18*). IMC-EB10 treatment of Hb1119 and SEM-K2 cell lines also decreased the phosphorylation of FLT3 and downstream signaling proteins compared with IMC-C225 (Fig. 1*B*, *lane 8* versus *lane 7, lane 11* versus *lane 10*). However, when IMC-EB10 was incubated with REH and RS411 cell lines, FLT3 and downstream pathways were activated, possibly by favoring receptor dimerization upon antibody binding (Fig. 1*B*, *lane 14* versus *lane 13, lane 17* versus *lane 16*).

IMC-EB10 significantly reduces the engraftment of SEM-K2 cells in NOD/SCID mice but not of low FLT3-expressing Karpas45 and NALM6 cells. We previously found that IMC-NC7

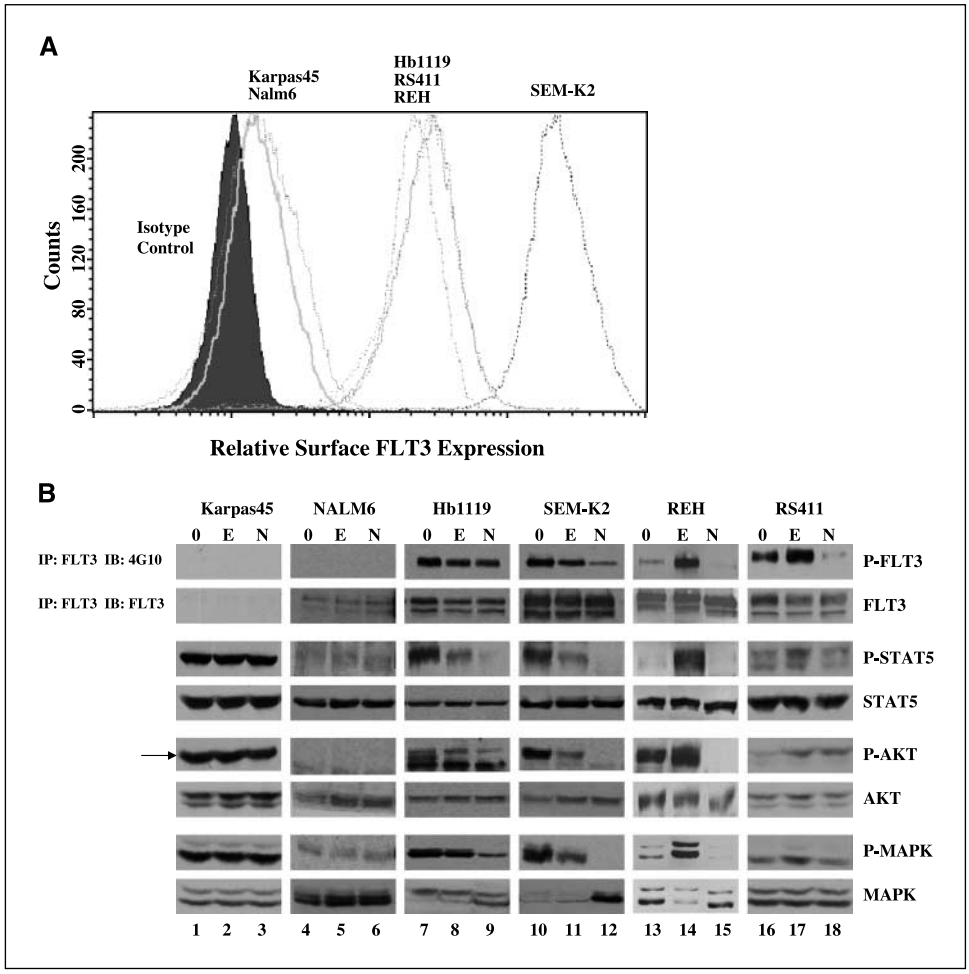

Figure 1. IMC-EB10 and IMC-NC7 can inhibit or activate FLT3 phosphorylation along with downstream STAT5, Akt, and MAPK signaling. *A,* ALL-derived cell lines were stained with CD135-PE or isotype control and analyzed by FACS. *B,* ALL-derived cell lines expressing wt FLT3 or FLT3-D835H (on Hb1119 cells) were treated with PBS (*0*), 10 μg/mL IMC-EB10 (*E*), or 10 μg/mL IMC-NC7 (*N*) for 1 hour. Immunoprecipitates and total protein extracts were resolved by 8% or 10% SDS-PAGE, respectively, and subjected to immunoblot analysis with the indicated phospho-specific antibodies. The same blots were then stripped and reprobed with protein-specific antibodies.

has no *in vivo* efficacy in animal leukemia models due to a reduced ability to mediate antibody-dependent, cell-mediated cytotoxicity (ADCC; ref. 32). Therefore, only IMC-EB10 was further tested *in vivo*. A variety of treatment schedules of IMC-EB10 were used for NOD/SCID mice injected with SEM-K2 cells. Mice were injected i.p. with IMC-EB10 thrice per week starting 24 hours after cell injection, once starting 24 hours after cell injection, a total of thrice every other day starting 24 hours after cell injection or thrice per week starting 7 days after cell injection. A group of mice were treated with control antibody (IMC-C225) i.p. thrice per week starting 24 hours after cell injection. After 30 days after cell injection, mice were killed, and peripheral blood, spleen, and bone marrow cells were analyzed by flow cytometry. Each of the IMC-EB10 treatment schedules resulted in a significant reduction of human cells in the peripheral blood, spleen, and bone marrow compared with IMC-C225 treatment (Fig. 2*A-C*). IMC-EB10 treatment typically reduced the presence of SEM-K2 cells to <1%. In contrast, IMC-EB10 treatment of NOD/SCID mice given REH or Hb1119 cells did not reduce leukemic engraftment (data not shown). IMC-EB10 also had no effect on reducing the engraftment of cell lines (Karpas45 and NALM6) expressing very low levels of FLT3 (data not shown).

IMC-EB10 prolongs survival of NOD/SCID mice given SEM-K2 cells. Mouse survival studies were done to determine if the reduced human engraftment shown in Fig. 2 would translate into improved survival. NOD/SCID mice given SEM-K2 cells were treated with IMC-C225 or IMC-EB10 using the same treatment regimens described previously. All IMC-EB10 treatment schedules resulted in significantly improved median survival compared with IMC-C225 treatment (Fig. 3*A* and *B*).

To determine if IMC-EB10 treatment had eliminated all ALL cells in the mice surviving 150 days after cell injection (which comprise 19% of IMC-EB10-treated mice), those mice were killed and assessed for the presence of human cells using flow cytometry and/or quantitative real-time PCR. Fluorescence-activated cell sorting (FACS) analysis show that five of the six mice treated with IMC-EB10 that survived 150 days after cell injection had no detectable human cells in their spleen or bone marrow above background (data not shown). Quantitative real-time PCR analysis of these mice revealed that with the exception of one mouse treated with IMC-EB10 once starting 24 hours after cell injection, the percentage of human cells in the spleen or bone marrow of these mice is ≤0.001%, which was the limit of detection in the assay (Fig. 3*C*). DNA was not available from one mouse treated with IMC-EB10 thrice per week and surviving until day 150.

IMC-EB10 reduces the engraftment of ALL primary cells in NOD/SCID mice. To assess the ability of IMC-EB10 to reduce leukemia *in vivo*, we used NOD/SCID mice injected with ALL patient samples and treated them with PBS, IMC-C225, or IMC-EB10 thrice per week starting 24 hours after cell injection via i.p. injections. After 3 to 4 months, leukemic cells found in NOD/SCID mice derive from transplanted leukemic stem cells because only

this small subpopulation of cells has the capability for sustained engraftment. After 3 to 4 months after cell injection, mice were killed, and the presence of human cells was determined by flow cytometry. IMC-EB10 treatments reduced the engraftment of two of four tested primary ALL samples in NOD/SCID mice; one of these samples is shown in Fig. 4*A*. Histologic analysis of the bone marrow from the IMC-EB10-treated mouse closest to the mean level of engraftment reveals normal appearing bone marrow with all lineages represented (Fig. 4*B*). In contrast, the bone marrow of mice treated with PBS or IMC-C225 are entirely replaced with sheets of leukemia cells. Western blot analysis of this ALL sample treated *in vitro* with IMC-EB10 shows activation of FLT3 and downstream Akt (Fig. 4*C*). Thus, activation of FLT3 by IMC-EB10 does not prevent the antibody from reducing leukemic engraftment *in vivo* (Fig. 4*A* and *C*).

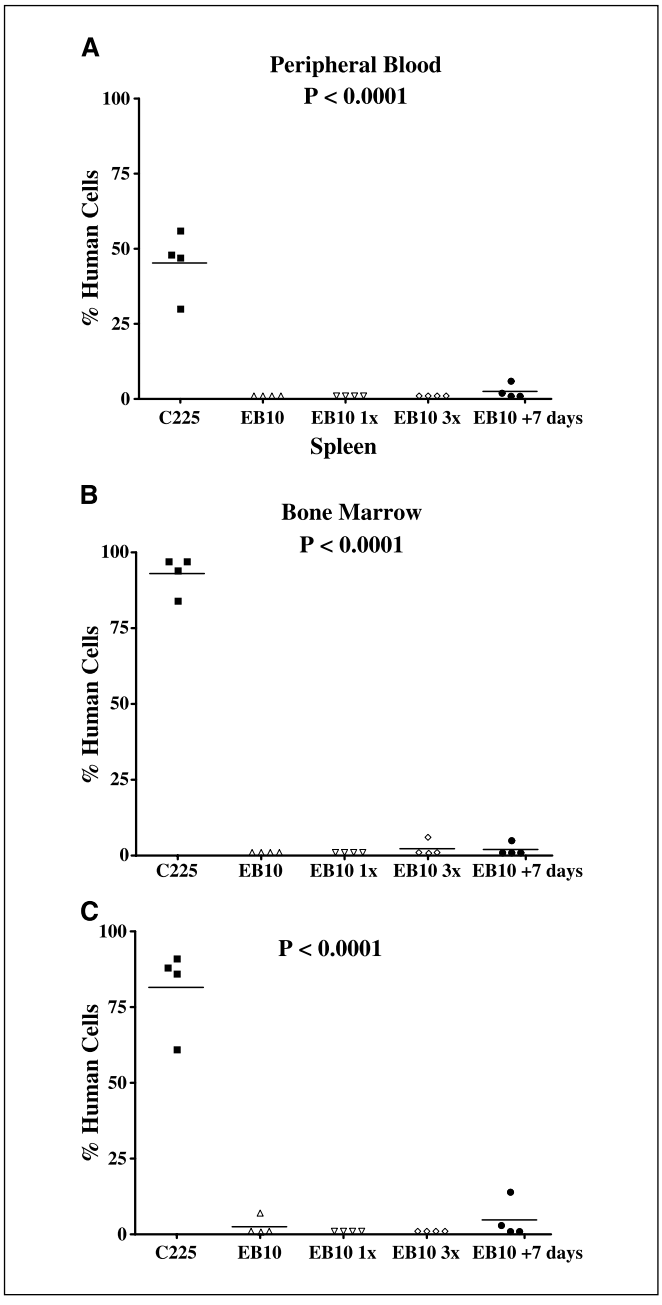

Figure 2. IMC-EB10 significantly reduces engraftment of SEM-K2 cells in NOD/SCID mice. NOD/SCID mice given 0.5×10^6 SEM-K2 cells via tail vein injection were injected i.p. with 400 μg IMC-C225 thrice weekly starting 24 hours after cell injection as control treatment or with 400 μg IMC-EB10 thrice weekly starting 24 hours after cell injection, once starting 24 hours after cell injection, thrice every other day starting 24 hours after cell injection, or thrice weekly starting 7 days after cell injection. Thirty days after cell injection, mice were killed, and the presence of human cells was determined by flow cytometry using human-specific CD19-FITC and CD45-APC antibodies: (*A*) peripheral blood, (*B*) bone marrow, and (*C*) spleen.

IMC-EB10 treatment does not select for resistant cells *in vivo*. As shown in Fig. 2, IMC-EB10 treatment of NOD/SCID mice given SEM-K2 cells results in almost undetectable levels of human engraftment 30 days after cell injection. However, most of these IMC-EB10-treated mice eventually die of leukemia with symptoms (i.e., weight loss, white marrow, and large spleen) identical to those of IMC-C225-treated mice (Fig. 3; data not shown). To determine if IMC-EB10 treatment selects for resistant cells, a NOD/SCID mouse given SEM-K2 cells and treated thrice per week with IMC-EB10 that displayed symptoms of morbidity was killed 120 days after cell injection. Bone marrow from this mouse, consisting of 85% SEM-K2 cells as determined by FACS, was retransplanted into NOD/SCID mice. As a control, SEM-K2 cells grown in culture and not exposed to IMC-EB10 were also injected into NOD/SCID mice. Mice were injected once with IMC-C225 or IMC-EB10 24 hours after cell injection. Mice were killed 30 days after cell injection, and the presence of human cells was determined by flow cytometry. A single injection of IMC-EB10 was shown to reduce leukemic engraftment of the spleen or bone marrow to undetectable levels, even when the injected SEM-K2 cells are those that survived in an IMC-EB10-treated mouse (Fig. 5*A* and *B*).

IMC-EB10 mediates cytotoxicity through natural killer cells *in vivo*. We previously showed that IMC-EB10 cytotoxicity is mediated *in vitro* through ADCC and requires natural killer (NK) cells. To investigate the role of NK cells in IMC-EB10-mediated cytotoxicity *in vivo*, we used Asialo GM1 antisera to reduce NK cell numbers *in vivo* (34–36). NOD/SCID mice were treated with an i.p. injection of 30 μL Asialo GM1 antisera + 70 μL PBS 3 days before and 4 and 11 days after SEM-K2 cell injection to suppress levels of NK cells. Pilot studies indicated that 3 days after a single injection of 30 μL Asialo GM1 antisera, NK cells in NOD/SCID mice were reduced by ~75% of their original level (data not shown). NOD/SCID mice injected with or without Asialo GM1 antisera were injected once i.p. with IMC-C225 or IMC-EB10 24 hours after SEM-K2 cells were injected. Thirty days after cell injection, mice were killed and assessed for the presence of human cells by flow cytometry. FACS analysis revealed that a reduction in NK cells numbers by Asialo GM1 antisera significantly impairs IMC-EB10-mediated cytotoxicity *in vivo* (Fig. 6*A*).

Because Fig. 6*A* shows that NK cells mediate IMC-EB10 *in vivo* cytotoxicity, we explored the possibility of improving IMC-EB10's efficacy by activating NK cells *in vivo*; i.p. administration of poly (I:C) has been shown to activate NK cells *in vivo*, and pilot studies confirmed this observation (data not shown; refs. 37, 38). Pilot studies indicated that a single i.p. injection of 125 μg poly (I:C) given 5 days after leukemic cells are injected into NOD/SCID mice does not significantly reduce the engraftment of human cells (data not shown). Therefore, NOD/SCID mice given RS411 cells were injected i.p. with three doses of IMC-EB10 given every other day starting 24 hours after cell injection and/or 125 μg poly (I:C) given i.p. 5 days after cell injection. Control mice were treated with IMC-C225 24, 72 and 120 hours after cell injection. Mice were killed

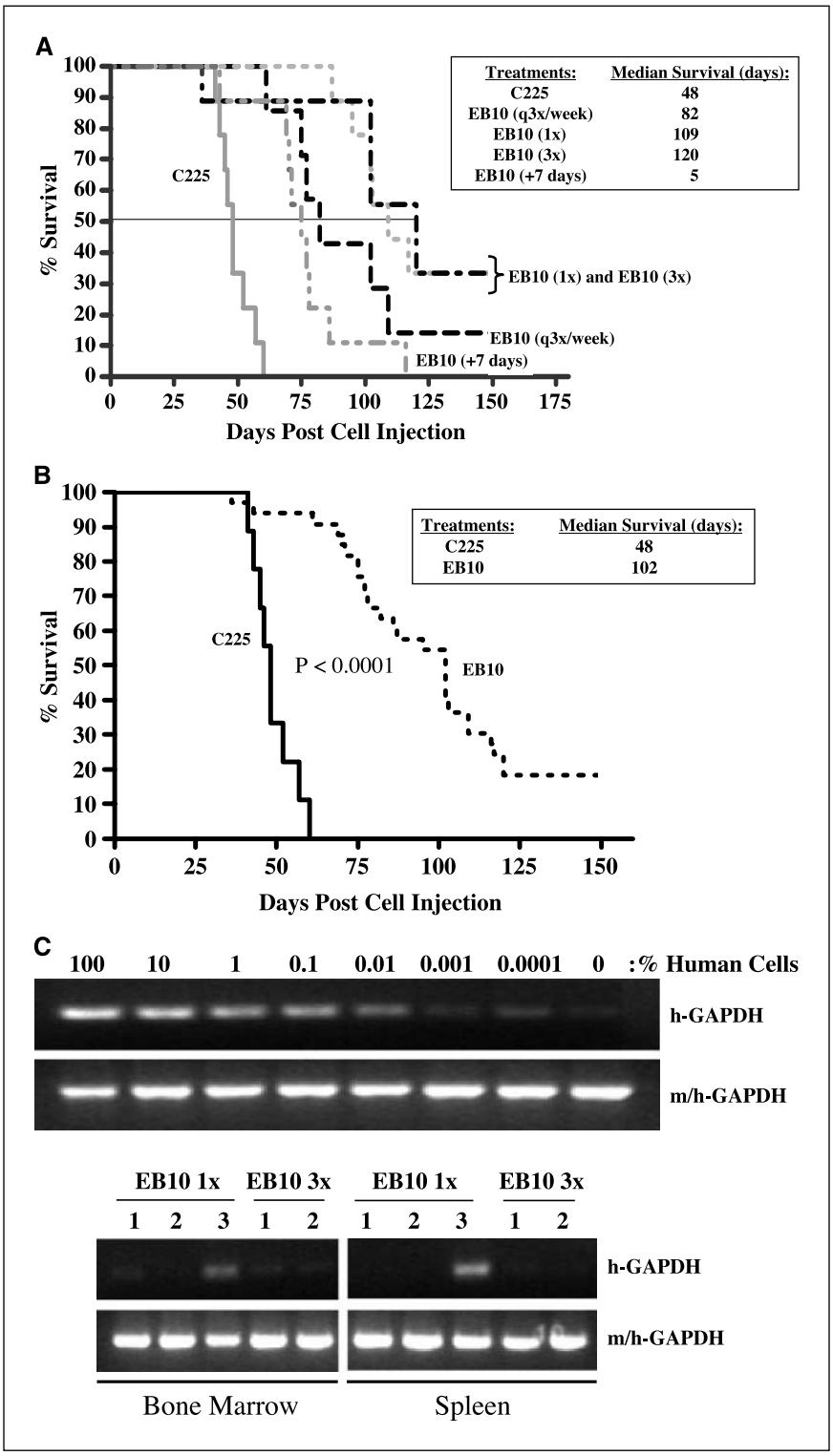

Figure 3. IMC-EB10 prolongs survival of NOD/SCID mice given SEM-K2 cells. NOD/SCID mice given 0.5×10^6 SEM-K2 cells via tail vein injection were injected i.p. with 400 μg IMC-C225 thrice weekly starting 24 hours after cell injection as control treatment or 400 μg IMC-EB10 thrice weekly starting 24 hours after cell injection, once starting 24 hours after cell injection, thrice every other day starting 24 hours after cell injection, or thrice weekly starting 7 days after cell injection. *A,* mice were monitored daily for survival. *B,* mouse survival data from all IMC-EB10 treatment regimens combined and compared with IMC-C225 treatment. *C,* mice surviving 150 days after cell injection were killed and bone marrow and spleen cells analyzed by quantitative real-time PCR to determine the presence of human cells. A standard curve, where murine spleen/bone marrow cells were mixed with SEM-K2 cells at fixed ratios, was used to estimate the percentage of human cells.

40 days after cell injection, and human engraftment was examined by flow cytometry. IMC-EB10 treatment significantly reduced the engraftment of RS411 cells in NOD/SCID mice compared with IMC-C225 or poly (I:C) treatments (Fig. 6*B*). Furthermore, combining IMC-EB10 treatment with poly (I:C) resulted in a further decrease in human engraftment. Thus, the *in vivo* efficacy of IMC-EB10 can be improved by activating NK cells.

Discussion

The high expression level of FLT3 on B-lineage ALL blasts and the roles that activated FLT3 plays in leukemogenesis give good credentials to FLT3 as a potential therapeutic target. A number of TKIs targeting FLT3 have been developed that possess both *in vitro* and *in vivo* efficacy, and several are being tested in clinical trials (23–27). Although FLT3 TKIs may prove useful in some clinical

settings, nonetheless, they have limitations as therapeutic agents targeting FLT3-expressing leukemias: (*a*) All known FLT3 TKIs also have activity against a spectrum of other kinases, which can result in some toxicities at the concentrations required to induce cytotoxicity. (*b*) Chronic treatment with TKIs can lead to resistance as has been seen with Gleevac (29). (*c*) In the majority of cases of AML and ALL, FLT3 is not mutated, and it is not clear how important its signaling contributes to survival and proliferation in these cells. A mAb approach targeting FLT3 has several potential advantages. Compared with TKIs, mAbs have the added advantage of being specific, and thus less toxic, and of recruiting the host's immune system in inducing apoptosis and could also prove effective in the setting of FLT3 mutants selected for resistance to TKIs from chronic exposure.

We previously reported that IMC-EB10 and IMC-NC7 are two mAbs that bind FLT3 and block FLT3 ligand binding (31, 32). IMC-EB10 was shown to mediate ADCC *in vitro* and reduce engraftment of an AML primary sample in NOD/SCID mice without affecting the engraftment of normal cord blood $CD34^+$ cells. In this report, we examined the *in vitro* and *in vivo* efficacy of using IMC-EB10 to treat ALL-derived cell lines and primary ALL samples and explored the mechanism employed by IMC-EB10 to induce cytotoxicity *in vivo*.

Leukemic cells consistently show up-regulated wt FLT3 expression/activation, although it is unclear how dependent they are on FLT3 signaling for survival (10–13). IMC-NC7 treatment consistently inhibited FLT3 phosphorylation, whereas IMC-EB10 treatment resulted in inhibition of FLT3 phosphorylation in some cell lines (Hb1119 and SEM-K2) but activation of FLT3 phosphorylation in others (REH and RS411). Both inhibition and activation of downstream phospho-STAT5, phospho-Akt, and phospho-MAPK correlated with FLT3 activation status in response to the antibodies. The bivalent nature of antibodies can result in the binding of two FLT3 monomers simultaneously, thus favoring receptor dimerization and activation. However, the ability of IMC-EB10 to induce FLT3 phosphorylation did not correlate with the level of surface FLT3 expression. The difference in the ability/inability of IMC-EB10 and IMC-NC7 to activate or inhibit FLT3 could result from differences in the epitopes recognized by each antibody.

Potentially, activation of FLT3 could provide a growth/survival advantage that could undermine attempts to induce cytotoxicity in leukemic cells. In any case, we could not find any evidence that either activation or inhibition of FLT3 signaling mediated by IMC-EB10 or IMC-NC7 treatment *in vitro* significantly affected ALL cell viability. One possible reason is that the antibodies may not reduce

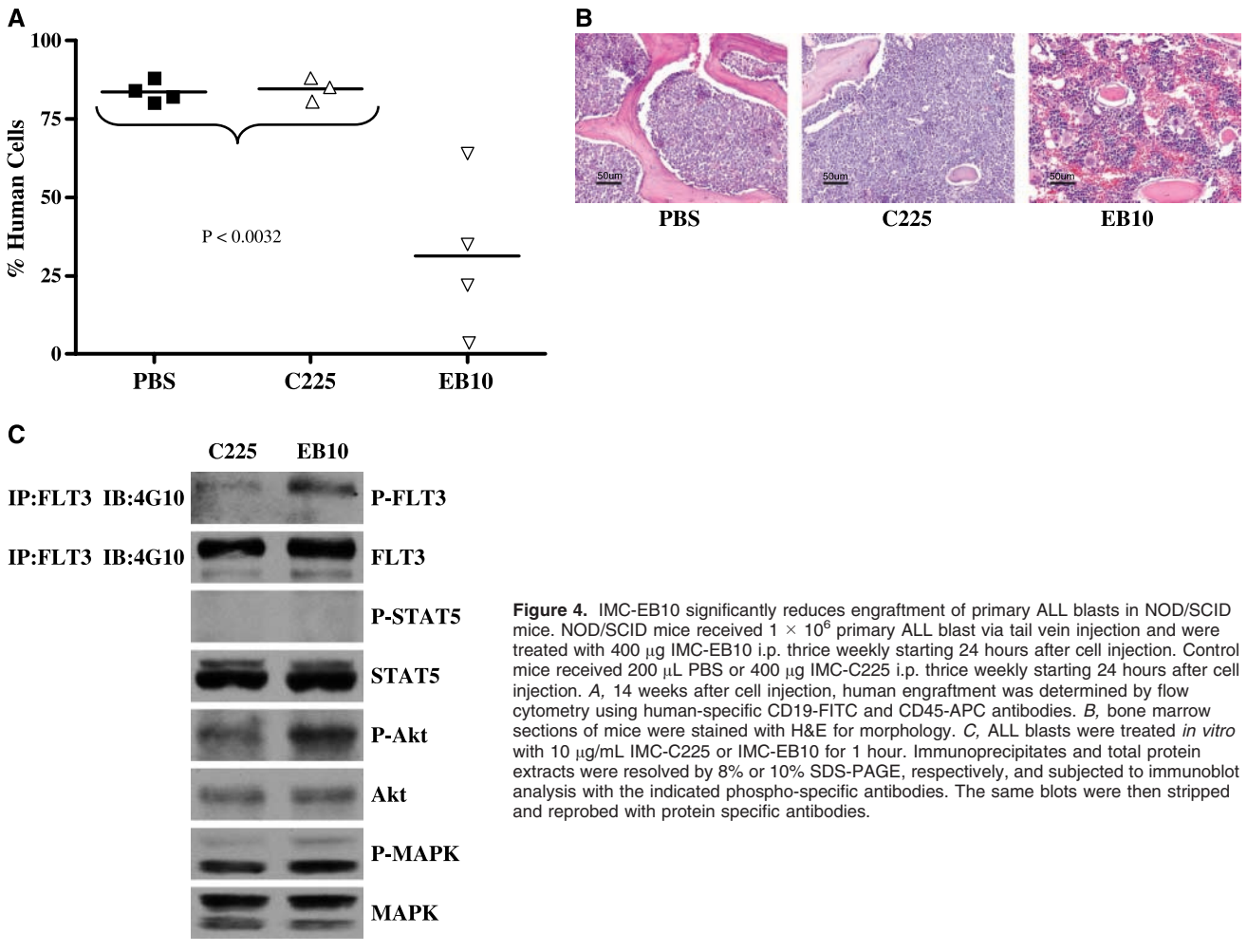

Figure 4. IMC-EB10 significantly reduces engraftment of primary ALL blasts in NOD/SCID mice. NOD/SCID mice received 1×10^6 primary ALL blast via tail vein injection and were treated with 400 μg IMC-EB10 i.p. thrice weekly starting 24 hours after cell injection. Control mice received 200 μL PBS or 400 μg IMC-C225 i.p. thrice weekly starting 24 hours after cell injection. *A,* 14 weeks after cell injection, human engraftment was determined by flow cytometry using human-specific CD19-FITC and CD45-APC antibodies. *B,* bone marrow sections of mice were stained with H&E for morphology. *C,* ALL blasts were treated *in vitro* with 10 μg/mL IMC-C225 or IMC-EB10 for 1 hour. Immunoprecipitates and total protein extracts were resolved by 8% or 10% SDS-PAGE, respectively, and subjected to immunoblot analysis with the indicated phospho-specific antibodies. The same blots were then stripped and reprobed with protein specific antibodies.

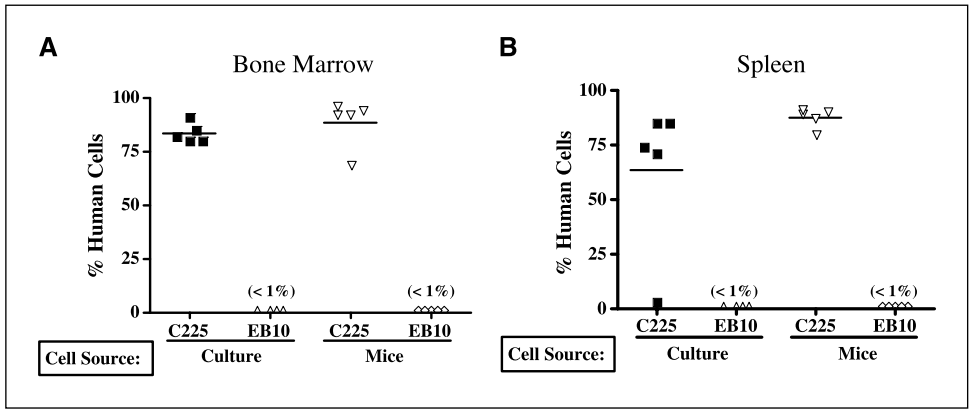

Figure 5. IMC-EB10 treatment of NOD/SCID mice given SEM-K2 cells do not select for resistant clones. NOD/SCID mice were injected via tail vein with 0.5×10^6 SEM-K2 cells grown in culture without IMC-EB10 or surviving IMC-EB10 treatment within NOD/SCID mice. Mice were injected once i.p. with 400 μg IMC-C225 or IMC-EB10 24 hours after cell injection. Thirty days after cell injection, mice were killed, and their (*A*) bone marrow and (*B*) spleen cells analyzed by flow cytometry for the presence of human cells using human-specific CD19-FITC and CD45-APC antibodies.

FLT3 phosphorylation beyond a critical threshold required to induce cytotoxicity. Even more likely, these ALL cell lines may not be completely dependent on FLT3 signaling for survival.

Although IMC-EB10 is not cytotoxic to ALL-derived cell lines *in vitro*, IMC-EB10 showed *in vivo* efficacy through ADCC. All IMC-EB10 treatment schedules significantly reduced the engraftment of SEM-K2 cells in NOD/SCID mice and prolonged their median survival compared with mice treated with a control antibody. The mice treated 7 days after leukemic injection had enhanced survival relative to control mice but shorter survival compared with mice whose treatment started 24 hours after injection. Reduced median survival due to delayed antibody treatment has also been observed with rituximab (39). This may be due to an increase in the ratio of leukemic cells to effector cells. Thus, combination treatment with chemotherapy first to reduce bulk disease may prove more effective.

Nineteen percent of the IMC-EB10-treated mice given SEM-K2 cells survived >150 days after cell injection. By quantitative real-time PCR and FACS analysis, those mice were found to have ≤0.001% human engraftment. Treatment with IMC-EB10 was also effective at reducing leukemic engraftment of two of four primary ALL samples at 3 to 4 months after leukemic cell injection, a time that reflects leukemic stem cell activity. This was true even when *in vitro* IMC-EB10 treatment resulted in activation of FLT3. This supports the hypothesis that IMC-EB10-mediated cytotoxicity is not dependent on inhibition of FLT3 signaling and is likely due to ADCC.

Although IMC-EB10 reduced the engraftment of several ALL-derived cell lines and primary samples, it was not efficacious against all cell lines and primary samples. IMC-EB10 treatment had no effect on NOD/SCID mice injected with two examples of low FLT3-expressing cells (Karpas45 and NALM6). This is not unexpected as a certain minimum level of surface FLT3 expression would be required to be effective, a phenomenon that has been observed with IMC-C225/EGFR and other antibodies (40). IMC-EB10 had no *in vivo* activity against the Hb1119 or REH cell lines nor against two of the four primary ALL samples that we tested, all of which express FLT3 at levels comparable with the cell lines and primary cells that were sensitive. The FLT3 activation status of these cells does not explain the inability of IMC-EB10 to kill these

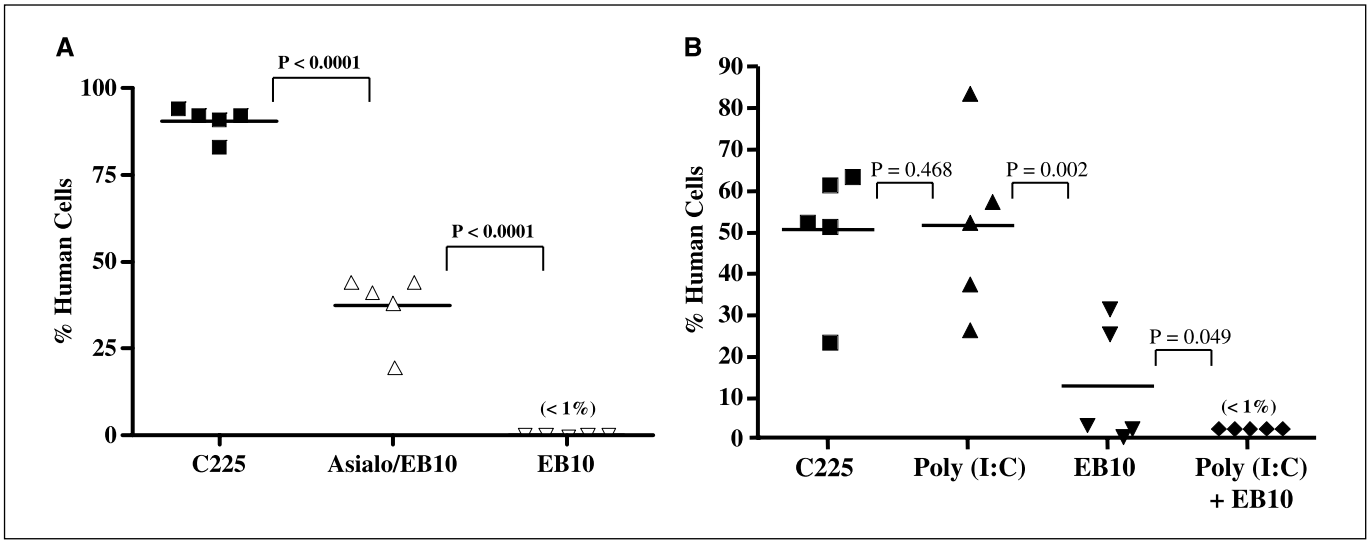

Figure 6. IMC-EB10 mediates cytotoxicity *in vivo* through NK cells. *A,* NOD/SCID mice were injected with 0.5×10^6 SEM-K2 cells via tail vein and treated with 400 μg IMC-EB10 IP 24 hours after cell injection and/or with 30 μL of Asialo GM1 antisera given at days −3, +4, and +11 relative to when SEM-K2 cells were injected. Control mice were injected with 400 μg IMC-C225 i.p. 24 hours after cell injection. Mice were killed 30 days after cell injection and analyzed for human engraftment using flow cytometry and human-specific CD19-FITC and CD45-APC antibodies. *B,* NOD/SCID mice were injected with 0.5×10^6 RS411 cells via tail vein and treated with three doses of 400 μg IMC-C225 or IMC-EB10 i.p. every other day starting 24 hours after cell injection. Five days after cell injection, 125 μg poly (I:C) was injected i.p. Forty days after cell injection, mice were killed and analyzed for human engraftment using flow cytometry and human-specific CD19-FITC and CD45-APC antibodies.

cells *in vivo* because IMC-EB10 was cytotoxic to an ALL primary sample and to RS411 cells *in vivo*, although *in vitro* IMC-EB10 treatment of these cells result in FLT3 activation. Expression of Fas ligand, MUC1, and/or tumor necrosis factor–related apoptosis-inducing ligand, which may have a role in blocking ADCC activity, has been associated with resistance to rituximab (41). However, FACS analysis of cell lines sensitive/resistant to IMC-EB10-mediated cytotoxicity did not show differential expression of these antigens. The more likely explanation would seem to be differences in their sensitivity to ADCC-induced apoptosis. The heterogeneous activity of IMC-EB10 has also been observed with other mAbs targeting CD20 or CD33 (42–45).

The development of resistance has been observed in patients treated with TKIs. Despite improved survival, most IMC-EB10-treated, SEM-K2-injected mice die, raising the possibility of selection of a resistant subpopulation of cells. However, retransplantation of leukemic cells surviving treatment showed they were still sensitive to IMC-EB10, indicating that treatment did not select for resistant clones.

One of the limitations of IMC-EB10 or any other mAb's efficacy in the NOD/SCID human leukemia model relates to the reduced NK cell activity of NOD/SCID mice (46). The multiple defects in innate immunity seen with NOD/SCID mice, thus, may underestimate the therapeutic potential of IMC-EB10. In addition, the *in vivo* efficacy of IMC-EB10 may be significantly greater in immune-competent hosts. We previously showed that NK cells are required for IMC-EB10-mediated *in vitro* ADCC against FLT3-expressing cells. In this report, we found that further decreasing the number of NK cells *in vivo* in NOD/SCID mice using Asialo GM1 antisera significantly interfered with the ability of IMC-EB10 to reduce the level of SEM-K2 cells. Moreover, activation of NK cells *in vivo* through administration of poly (I:C) improved the ability of IMC-EB10 to reduce the level of RS411 cells in NOD/SCID mice. Thus, these experiments further implicate NK cells as a key component in IMC-EB10-mediated cytotoxicity. *In vivo* activation of NK cells may well be a feasible method for improving the efficacy of IMC-EB10 against FLT3-expressing leukemias. In addition, IMC-EB10-mediated cytotoxicity will likely be less dependent on the host's immune system if IMC-EB10 is conjugated to a radionuclide or toxin as has been shown for a variety of antibodies (47).

In conclusion, the data show that IMC-EB10 reduces the engraftment and improves the survival of NOD/SCID mice injected with some ALL-derived cell lines and primary ALL samples. NK cell activity is a critical factor mediating IMC-EB10 cytotoxicity. Anti-FLT3 mAbs, like IMC-EB10, may prove efficacious against a wide range of FLT3 expressing malignancies, which include ALL, AML, and subsets of CML blast crisis, T-ALL, and chronic lymphocytic leukemia. These mAbs may also prove useful against TKI-resistant cells because their mode of action does not depend on inhibition of FLT3 signaling. Thus, mAbs targeting FLT3 are a specific and potentially effective therapeutic agent that should be further investigated and considered for clinical trials.

Acknowledgments

Received 1/6/2006; accepted 2/16/2006.

Grant support: National Cancer Institute grants CA90668, CA70970, and CA100632 (D. Small); Leukemia and Lymphoma Society (D. Small); Burroughs Wellcome Trust (D. Small); and Grants RR00171 and CA62924 (D. Huso).

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