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Monoclonal Antibody-Mediated Targeting of CD123, IL-3 Receptor α Chain, Eliminates Human Acute Myeloid Leukemic Stem Cells

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SUMMARY

Leukemia stem cells (LSCs) initiate and sustain the acute myeloid leukemia (AML) clonal hierarchy and possess biological properties rendering them resistant to conventional chemotherapy. The poor survival of AML patients raises expectations that LSC-targeted therapies might achieve durable remissions. We report that an anti-interleukin-3 (IL-3) receptor α chain (CD123)-neutralizing antibody (7G3) targeted AML-LSCs, impairing homing to bone marrow (BM) and activating innate immunity of nonobese diabetic/severe-combined immunodeficient (NOD/SCID) mice. 7G3 treatment profoundly reduced AML-LSC engraftment and improved mouse survival. Mice with pre-established disease showed reduced AML burden in the BM and periphery and impaired secondary transplantation upon treatment, establishing that AML-LSCs were directly targeted. 7G3 inhibited IL-3-mediated intracellular signaling of isolated AML CD34$^+$ CD38$^-$ cells in vitro and reduced their survival. These results provide clear validation for therapeutic monoclonal antibody (mAb) targeting of AML-LSCs and for translation of in vivo preclinical research findings toward a clinical application.

INTRODUCTION

The 5 year survival rate of patients under 60 years of age with AML is less than 30%, with progressively worse prognosis for more elderly patients (Estey and Dohner, 2006). The cellular and molecular basis for this dismal picture is unclear; however, a large body of work is emerging in experimental systems that predicts LSCs may lie at the heart of posttreatment relapse and chemoresistance. AML is organized as a cellular hierarchy sustained by LSCs at their apex (Bonnet and Dick, 1997; Guan and Hogge, 2000; Guzman et al., 2001; Hope et al., 2004; Lapidot et al., 1994; Wang and Dick, 2005). LSCs are the only AML cells capable of self-renewal while still generating rapidly proliferating progenitors and terminal leukemic blasts. The rare occurrence, generally dormant nature, and abnormal apoptotic response via the NF-κB pathway of LSCs are all properties that may render them resistant to conventional chemotherapeutics that target proliferating cells (Bonnet and Dick, 1997; Guan and Hogge, 2000; Guzman et al., 2001; Hope et al., 2004; Ishikawa et al., 2007; Lapidot et al., 1994; Wang and Dick, 2005). In addition, minimal residual disease occurrence and poor survival have been attributed to the ability of LSCs to engraft NOD/SCID mice (Pearce et al., 2006) and high CD34$^+$CD38$^-$ frequency at time of diagnosis in AML patients (van Rhenen et al., 2005). Consequently, it is imperative that new treatments are developed to complement established chemotherapy by specifically eliminating AML-LSCs for the long-term management of the disease (Abutalib and Tallman, 2006; Aribi et al., 2006; Morgan and Reuter, 2006; Stone, 2007).

As with normal hematopoietic stem cells (HSCs), very little is known of the molecular regulation that governs the self-renewal, differentiation, and survival of AML-LSCs, although both of these stem cell types share the properties of slow division, self-renewal ability, and expression of some surface markers including the CD34$^+$CD38$^-$ immunophenotype (Bhatia et al., 1997; Bonnet and Dick, 1997; Lapidot et al., 1994). CD123, which is widely reported to be overexpressed on AML blasts, CD34$^+$ leukemic progenitors, and AML-LSCs in comparison with normal HSCs (Florian et al., 2006; Graf et al., 2004; Hauswirth et al., 2007; Jordan et al., 2000; Munoz et al., 2001; Riccioni et al., 2004; Sperr et al., 2004; Testa et al., 2002; Yalcintepe et al., 2006), represents a promising cell-surface target for the development of therapeutics that specifically target AML-LSCs but not HSCs. CD123 is the α subunit of the IL-3 receptor (IL-3R), the major binding protein for IL-3, which together with CD131 (β_c) forms the functional heterodimeric high-affinity IL-3R. The binding of IL-3 to CD123 is species specific and leads to activation of the receptor that promotes cell survival and proliferation (Bagley et al., 1997; Miyajima et al., 1993).

Overexpression of CD123 on AML cells confers a range of growth advantages over normal HSCs; AML cells proliferate

extensively with IL-3 treatment in vitro (Budel et al., 1989; Miyauchi et al., 1987; Pebusque et al., 1989; Vellenga et al., 1987), and some AML samples secrete cytokines including IL-3 (Elbaz and Shaltout, 2001; Guan et al., 2003; Nowak et al., 1999). Moreover, high-level CD123 expression on AML cells correlates with the level of IL-3-stimulated and spontaneous signal transducer and activator of transcription 5 (STAT5) activation, the proportion of cycling cells, a more primitive cell-surface phenotype, and resistance to apoptosis (Graf et al., 2004; Testa et al., 2002, 2004). Clinically, high CD123 expression in AML is associated with higher blast counts at diagnosis and a lower complete remission rate that results in reduced survival (Graf et al., 2004; Testa et al., 2002, 2004). Collectively, these data point to the significance of CD123 expression in leukemia cell stimulation and AML patient outcome.

The increased expression of CD123 on LSCs compared with HSCs presents an opportunity for selectively targeting AML-LSCs with a therapeutic antibody. Besides the possibility that IL-3 is required for LSC functions, an antibody to CD123 could stimulate host immune-mediated mechanisms for cell killing. An antibody with both IL-3R-neutralizing and innate immunity-activating properties could represent an ideal therapeutic candidate for clinical testing. The mAb 7G3, raised against CD123, has previously been shown to inhibit IL-3-mediated proliferation of leukemic cell lines (Sun et al., 1996). While AML-LSCs are often cited to be enriched in the $CD34^+CD38^-$ fraction, recent reports have demonstrated that other fractions, such as the $CD34^+CD38^+$ subpopulation, also have NOD/SCID repopulating capacity (McKenzie et al., 2006; Taussig et al., 2008). In this report, we show that CD123 is highly expressed on the bulk of AML cells as well as the $CD34^+CD38^-$ fraction compared to normal hematopoietic cells. Importantly, we demonstrate that 7G3 targeting of CD123 in the absence of exogenous human cytokines impairs AML-LSCs in vivo. This occurs through at least two mechanisms involving inhibition of homing of $CD34^+CD38^-$ cells and engraftment of AML-LSCs in the NOD/SCID xenograft model, as well as activation of innate immunity in NOD/SCID mice. As a prerequisite for the potential role of 7G3 in inhibiting IL-3-mediated growth advantages on AML-LSCs, we demonstrate that both the unsorted and the $CD34^+CD38^-$ subpopulations of AML cells proliferate and survive via IL-3-mediated intracellular signaling pathways and that these are inhibited by 7G3 in vitro. The recent characterization of defined populations of cancer stem cells (CSCs) in a range of human malignancies (Wang, 2007), as well as their relative resistance to conventional chemotherapy and radiotherapy (Rich and Bao, 2007), supports the broad applicability of our approach and provides rationale for the progression of AML-LSC-targeted therapeutics from preclinical evaluation to clinical trials.

RESULTS

Ex Vivo 7G3 Treatment Selectively Inhibits AML Engraftment in NOD/SCID Mice

Since AML-LSCs are central to long-term AML growth and they are difficult to assay in vitro, we used the SCID-leukemia initiating cell (SL-IC) assay to determine whether 7G3 can directly target AML-LSCs and inhibit their repopulating ability. Ex vivo 7G3 incubation markedly reduced the engraftment of 10 of 11 primary AML samples in sublethally irradiated NOD/SCID mice to a mean of 11.4% ± 1.9% of isotype-matched (IgG2a-treated) controls ($p = 0.00021$, Figure 1A, Table 1). This reduction in engraftment was sustained in five of seven samples when assessed between 8 and 10 weeks following inoculation (5.7% ± 1.7% of controls, $p = 0.004$). Ex vivo 7G3 treatment inhibited the engraftment of AML-8 harvested at both diagnosis and relapse to a similar extent. AML-5 was the only AML sample in which engraftment was not reduced by ex vivo 7G3 treatment. Although the reason for this is unknown, it is noteworthy that AML-5 is a monosomy 7 sample (noted for poor prognosis) with relatively low CD123 expression (Table 1).

We next investigated the sensitivity of normal cord blood (CB) and BM (NBM) to 7G3 using the same strategy as for AML samples to determine if there was differential targeting of normal HSCs. When measured at 4–11 weeks postinoculation, 7G3 significantly reduced the engraftment of only two of five normal samples (Figure 1B and Table 1). The inhibitory effect of 7G3 on the engraftment of normal cells (76.5% ± 8.9% engraftment relative to IgG2a controls) was significantly less ($p < 0.0001$) than against AML cells. Additionally, 7G3 treatment did not alter the differentiation profiles of the engrafted normal human hematopoietic populations (data not shown). Furthermore, to demonstrate the clinical relevance and specificity of 7G3 treatment against LSCs and not normal HSCs, we showed that a mouse anti-human HLA-A,B,C antibody indiscriminately inhibited the engraftment of two AML and three normal samples (see Figure S1 available online). Independent analysis at two different institutions (Sydney and Toronto) revealed that CD123 expression on AML $CD34^+CD38^-$ cells (relative fluorescence index [RFI] 38.2 ± 6.6) was significantly higher than on their normal counterparts (RFI 9.6 ± 1.6) (Figure 1C and Table 1). The engraftment levels of ex vivo 7G3-treated samples were inversely correlated with the intensity of CD123 expression on the $CD34^+CD38^-$ population (Figure S2; Spearman $R = -0.69$). Taken together, we can conclude that normal HSCs are considerably less sensitive to 7G3 than AML-LSCs, due, at least in part, to their relatively low levels of cell-surface CD123 expression.

The reduction in AML engraftment caused by ex vivo 7G3 treatment was also associated with improved survival. Mice transplanted with IgG2a- or 7G3-treated AML-9 cells exhibited median survival of 11.5 and 24 weeks, respectively (Figure 1D), with 40% of the 7G3 group surviving beyond the end of the experiment (25 weeks), in contrast with the control group, in which no mice survived beyond 20 weeks.

7G3 Inhibits AML Homing Capacity in NOD/SCID Mice

To gain insight into the mechanism whereby 7G3 inhibited AML-LSC engraftment, we investigated the influence that antibody binding had on AML cell trafficking, since the SL-IC assay requires AML-LSCs to traffic to the BM in order to survive and proliferate, thereby establishing a leukemic graft. Homing assays were performed on two AML samples (AML-8-rel and -9) following ex vivo 7G3 treatments. 7G3 reduced the homing efficiency of AML-9 in the BM to 12.2% ± 2.7% and in the spleen to 9.4% ± 2.4% of controls (Figure 2A), and inhibited the homing of AML-8-rel in the BM to 34.7% ± 5.6% (Figure 3A) and in the spleen to 46.9% ± 3.5% of controls. To better distinguish the

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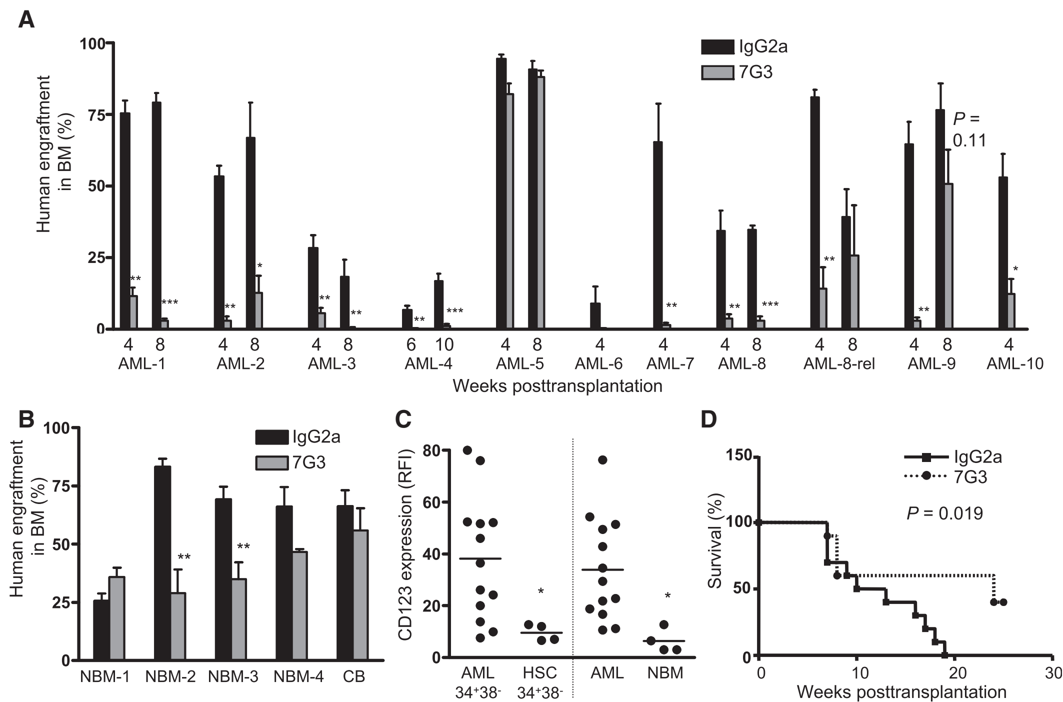

Figure 1. Ex Vivo 7G3 Treatment Selectively Inhibits the Repopulating Ability of AML Primary Cells in NOD/SCID Mice

(A) Percentage of human AML cells in the BM of mice transplanted with 7G3 or IgG2a control-treated AML cells at indicated time points. n = 3–10 per treated group.

(B) Levels of human engraftment in the BM of mice transplanted with 7G3 or IgG2a control-treated CB and NBM cells. Bars of CB represent the results from three separate experiments. n = 4–6 mice per group in each experiment.

(C) CD123 expression on total and $CD34^{+}CD38^{-}$ fractions of AML and normal cells. Each point represents an individual sample. Bars represent the mean.

(D) Kaplan-Meier survival curves of mice transplanted with IgG2a or 7G3 ex vivo-treated AML-9 cells. Survival curves were compared by log rank test. n = 10 per group. Error bars represent mean ± SEM; *p < 0.05, **p < 0.01, and ***p ≤ 0.0001 between selected groups.

effects of 7G3 on AML homing, lodgment, and proliferation, ex vivo-treated AML-8-rel cells were transplanted intravenously (i.v.) via the tail vein or directly into the right femur (RF). The intrafemoral (IF) approach circumvents the AML-LSC trafficking/homing processes associated with the circulation (Mazurier et al., 2003). While 7G3 remained effective in significantly reducing the engraftment in both the injected femur and the non-injected bones, IF inoculation did attenuate the inhibitory effects of 7G3 on engraftment in comparison with i.v. inoculation (Figure 2B).

In order to more directly demonstrate 7G3 inhibition of AML-LSCs, we investigated the impact of 7G3 treatment on $CD34^{+}CD38^{-}$ cells since AML-LSCs are significantly enriched in this fraction (Bonnet and Dick, 1997). The number of $CD34^{+}CD38^{-}$ cells from AML-8-rel and AML-9 homing to the BM was reduced by ex vivo 7G3 treatment to 8.4% ± 0.018% and 12.0% ± 4.3% of control, respectively (Figure 2C). Similarly, the number of AML-9 $CD34^{+}CD38^{-}$ cells homing to the spleen was reduced to 3.8% ± 1.5% of control. To further confirm this finding, the homing assay was repeated with sorted $CD34^{+}CD38^{-}$ cells from AML-9 following ex vivo antibody treatment. The homing efficiency of human cells in the 7G3-treated group was reduced to 7.8% ± 1.7% of IgG2a controls in the BM and 11.2% ± 0.84% in the spleen (Figure 2D). Consistent with the observation that 7G3 diminished AML-LSC homing capacity, ex vivo 7G3 treatment reduced the number of $CD34^{+}CD38^{-}$ cells in the BM xenografts of three AML samples (Figure 2E). By contrast, the number of $CD34^{+}CD38^{-}$ cells present in xenografts established from four independent normal hematopoietic samples following ex vivo 7G3 treatment was 81.9% ± 11.6% of IgG2a controls (p = 0.19, data not shown). Collectively, we can conclude that 7G3 inhibits not only homing but also lodgment and proliferation of AML-LSCs in the BM microenvironment.

7G3-Mediated Inhibition of AML-LSC Homing and Engraftment Is Fc Dependent

In order to determine whether the inhibitory effects of 7G3 are Fc mediated, the homing efficiency of AML cells was examined following treatment with F(ab′)2 fragments of various CD123-targeting antibodies. Incubation of AML-8-rel with two MAbs clones, 6H6 and 9F5, that bind CD123 but are weakly neutralizing reduced the homing efficiency in the BM to a similar extent as 7G3 (Figure 3A). In contrast, when AML-8-rel cells were treated ex vivo with 7G3 or 6H6 F(ab′)2 fragments, the inhibitory effects of each antibody on AML homing were attenuated.

In addition, the Fc requirement for inhibition of NOD/SCID repopulation was also examined. While ex vivo incubation of AML-9 and AML-10 cells with 7G3 or 9F5 significantly reduced their ability to repopulate mouse BM, the corresponding F(ab′)2

Table 1. Patient Characteristics, CD123 Expression, and Effects of mAb 7G3 on Engraftment of AML and Normal Hematopoietic Cells

Cells Transplanted	Age/ Sex	AML Subtype	Cytogenetics	Flt3 Mutational Status	WCC	Current Clinical Status	Sample Type	Overall Survival (Days)	$CD34^+$ $CD38^-$ (%)	CD123 (RFI)	Effect of 7G3 (Engraftment as % Control)
AML											
1	69/F[a]	M0[b]	Normal	Mutant	231[a]	Deceased	Apheresis	288[c]	2.9[d]	52.1[e]	15.2[f]
2	70/F	M1	Normal	Mutant	270	Deceased	Apheresis	196	2.2	26.1	5.3
3	64/F	M5b	Normal	Wild-type	80	Deceased	BM	1378	0.048	9.9[g]	19.5
4	75/M	M5a	Trisomy 8	Mutant	300	Deceased	Apheresis	27	3.5	36.5	4.7
5	19/F	M2	Maturation/ monosomy 7	NA	108	Deceased	NA	NA	6.2	13.8	97.1
6	53/M	M4Eo	Inv16	Mutant	300	Deceased	BM	504	4.9	24.2	1.5
7	80/M	M5	NA	NA	122	NA	Apheresis	45	1.9	80	2
8	47/F	M4	NA	NA	33	Deceased	Apheresis	436	8.28	46	11.1
8—Relapse	47/F	M4	NA	NA	33	Deceased	Apheresis	436	6.6	52.4	17.3
9	55/F	M5a	46XX	NA	161	NA	Apheresis	4	35.1	76.1	1.4
10	80/F	M2	46XX	NA	130	NA	Apheresis	NA	38.5	7.6	23.2
11	78/M	M2	Normal	Wild-type	166	Deceased	BM	26	0.2	51.7[g]	NE
12	67/M	M5b	Normal	Mutant	212	Deceased	BM	2	0	20[g]	NE
Normal											
NBM-1	26/M	–	–	–	–	–	BM	–	0.4	12	139.9
NBM-2	35/M	–	–	–	–	–	BM	–	2.3	6.7	34.8
NBM-3	32/M	–	–	–	–	–	BM	–	0.4	7	50.4
NBM-4	NA	–	–	–	–	–	BM	–	NA	NA	70.3
CB-1	NA	–	–	–	–	–	CB	–	NA	12.7	79.1

NBM-3 was a $CD34^+$ sorted normal BM sample. BM, bone marrow. CB, cord blood. NA, not available. NE, no engraftment in controls. WCC, peripheral blood white cell count ($\times 10^9$/L).
[a] At diagnosis (this column).
[b] FAB criteria (this column).
[c] From date of initial diagnosis (this column).
[d] Percent of total population (this column).
[e] RFI of $CD34^+CD38^-$ population (this column).
[f] Mean engraftment in the ex vivo 7G3-treated group as a percentage of the IgG2a-treated group, based on Figures 1A and 1B (this column).
[g] Sample had very low proportion of $CD34^+$ cells.

antibody fragments were ineffective (Figure 3B), despite 7G3 F(ab′)2 retaining its IL-3Rα-neutralizing activity (data not shown). The requirement for Fc regions to inhibit homing and repopulation, combined with the reduced efficacy of 7G3 when trafficking in the circulation was circumvented by IF transplantation, strongly supports a role for the innate immune system in mediating at least a portion of the inhibitory effects of 7G3.

$CD122^+$ Cells Contribute to 7G3-Mediated Inhibition of AML Homing and Repopulation in NOD/SCID Mice

While NOD/SCID mice are devoid of functional T and B cells, and are defective in complement fixation, they retain residual levels of innate effector activity (principally due to NK cells and macrophages) that can affect stem cell engraftment. To determine whether residual NOD/SCID innate immunity contributed to the inhibitory effects of 7G3 on LSCs, mice were injected with anti-CD122 mAb prior to transplantation with ex vivo 7G3-treated AML-8-rel cells. In the IgG2a control-treated groups, leukemic engraftment in the $CD122^+$ cell-depleted mice was increased to 113.3% ± 2.8% of nondepleted mice (Figure 3C), reflective of our earlier data showing increased detection of HSCs in such recipients (McKenzie et al., 2005). We found that depletion of $CD122^+$ cells significantly attenuated the ability of 7G3 to reduce leukemia engraftment from 82.7% ± 9.4% to 39.8% ± 14.3% inhibition relative to control antibody (Figure 3C), although a significant difference still remained between 7G3-treated and IgG2a-treated groups. Similarly, when we used NOD/SCID interleukin-2 receptor γ chain null mice (NOD/SCID/IL-2Rγ^{null}), which have lower residual NK cell activity than NOD/SCID mice (Ito et al., 2002), we observed similar attenuation, but not complete ablation, of the inhibitory effects of ex vivo 7G3 treatment on AML-1 engraftment in the BM (33.3% ± 12.6% of control compared with 1.1% ± 0.9% of control for NOD/SCID mice, Figure S3).

Additionally, anti-CD122 antibody treatment also partially attenuated the ability of 7G3 to block AML cell homing to the BM observed in both AML-8-rel and AML-9. As shown in Figure 3D, the homing efficiency of AML-9 cells treated with 7G3 was 8.4% ± 1.4% of control, and this was attenuated to 18.2% ± 3.1% with depletion of $CD122^+$ cells. The number of

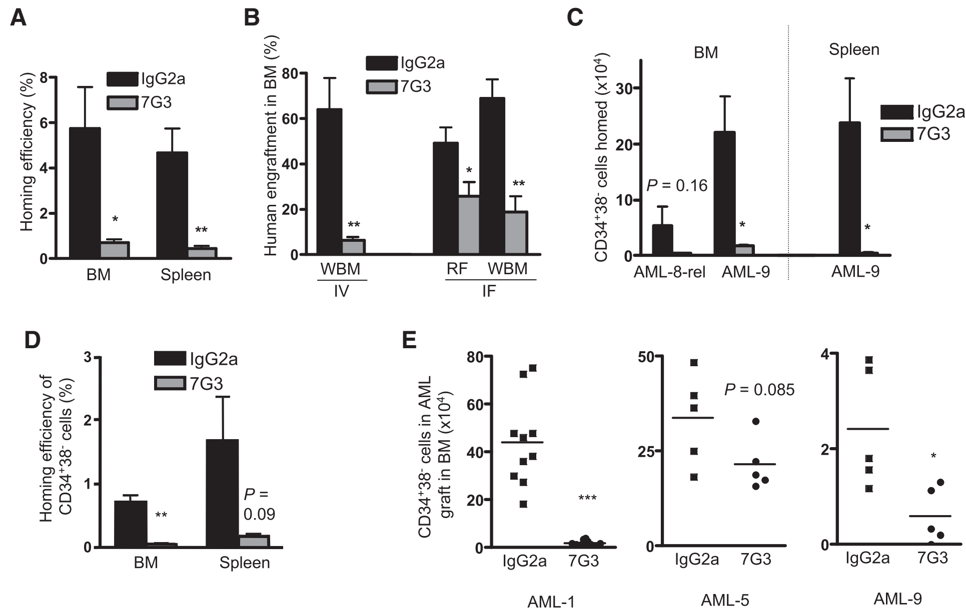

Figure 2. Inhibition of AML-LSC Homing Contributes to the Inhibitory Efficacy of 7G3

(A) Homing efficiency of AML-9 cells to the BM and spleen of mice following ex vivo 7G3 treatment from two separate experiments. n = 3–6 per group.

(B) Engraftment of ex vivo antibody-treated AML-8-rel cells in the injected femur (RF) and whole BM (WBM) after i.v. (IV) or intrafemoral (IF) transplantation. n = 4–5 mice per group.

(C) Absolute number of $CD34^+CD38^-$ AML cells homed in the BM and spleen of NOD/SCID mice injected with ex vivo 7G3-treated leukemic cells. n = 2–3 or 5 mice per group for AML-8 and AML-9, respectively.

(D) Homing efficiency of sorted $CD34^+CD38^-$ AML-9 cells after ex vivo treatment into both BM and spleen of mice. n = 3 mice per group.

(E) The number of $CD34^+CD38^-$ cells in the AML graft of mouse BM transplanted with AML-1, -5, and -9 after ex vivo IgG2a or 7G3 treatment. Each symbol represents a single mouse; horizontal bars indicate the mean. Error bars represent mean ± SEM; *$p < 0.05$, **$p < 0.01$, and ***$p \leq 0.0001$ between IgG2a and 7G3 groups.

$CD34^+CD38^-$ cells that homed to the BM of mice was also reduced to 5.3% ± 1.1% of control (Figure 3E), and this number was only marginally increased by the addition of anti-CD122 antibody (8.2% ± 1.9% of control, Figure 3E).

Collectively, our results indicate that the ability of 7G3 to inhibit engraftment and homing of AML cells in NOD/SCID mice is mediated by at least two cooperative pathways: immune effector activity caused by NK and/or other $CD122^+$-dependent cells, and specific inhibitory effects of 7G3 on AML-LSC homing and engraftment.

7G3 Reduces AML Burden in NOD/SCID Mice

Several in vivo treatment strategies were adopted to determine whether direct injection of 7G3 into NOD/SCID mice affected AML engraftment: (1) administering 7G3 to the mice 6 hr before cell transplantation almost completely ablated AML-1 engraftment in mouse BM to 1.3% ± 0.9% of IgG2a control at 5 weeks posttransplantation (Figure 4A); (2) initiating 7G3 treatment at 24 hr posttransplantation, to allow for LSC homing, also reduced the engraftment of two of three AML samples at 5 weeks posttransplantation (Figure 4B), indicating that early administration of 7G3, when the leukemic burden is low, can efficiently impair the engraftment of AML cells in NOD/SCID mice; (3) commencing 7G3 or IgG2a administration 28 days posttransplantation, in an established disease model, and continuing treatment until time of sacrifice, a significant reduction in the BM burden of AML was seen in two of five samples, likely reflective of the heterogeneity of AML seen clinically. AML-2 responded to 7G3 with reductions in BM engraftment at 9 and 14 weeks posttransplantation (Figure 4C), while treatment of mice with only four doses of 7G3 over 8 days significantly reduced the engraftment of AML-1 (Figure 4D). Moreover, while some AML samples did not have a significant reduction in leukemic burden in the BM with initiation of 7G3 treatment at either 4 or 28 days posttransplantation, a significant reduction in AML burden in the liver and spleen, but not the peripheral blood, was observed (Figures 4E–4G).

To further assess the clinical potential of a CD123-targeting mAb, 7G3 treatments were commenced in mice at 35 days posttransplantation with NBM cells and caused no significant reduction in BM infiltration when administered for 8 days or continuously for 5 weeks (Figure 4D and data not shown). Moreover, 7G3 caused no significant impairment of multilineage engraftment of normal cells (data not shown). Together, these data suggest that 7G3 is biologically active in vivo and can repress the growth of AML with lesser effects on normal human hematopoietic cells.

Since murine NOD/SCID cells do not bind 7G3, we carried out preclinical toxicity studies in a more relevant large animal model, the cynomolgus monkey. A chimeric variant of mAb 7G3 was engineered that maintains CD123 binding specificity and neutralization activity reformatted with a human IgG_1 Fc region. This model permits evaluation of any effect on resting hematopoiesis where there is a source of endogenous IL-3 and a normal immune system. The mAb was administered by i.v. infusion once

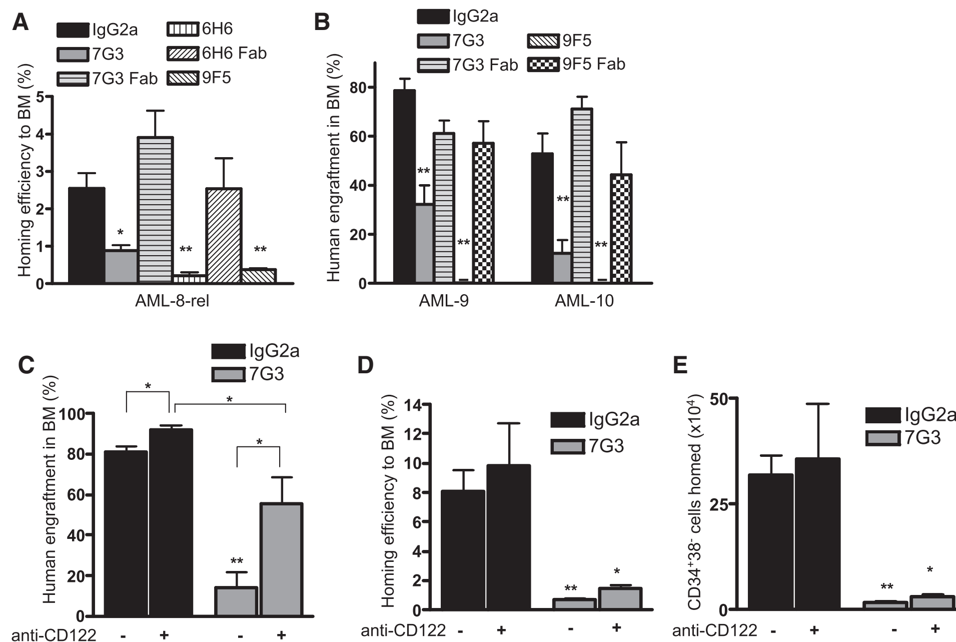

Figure 3. Fc Region of the Antibody and Innate Immunity Mediate 7G3 Antileukemic Effects

(A) Homing efficiency of AML-8-rel cells to the BM following ex vivo treatment with IgG2a, 7G3, 7G3 F(ab′)2, 6H6, 6H6 F(ab′)2, or 9F5. n = 3 per group.

(B) Engraftment of AML-9 and -10 in the BM of mice following ex vivo IgG2a, 7G3, 7G3 F(ab′)2, 9F5, or 9F5 F(ab′)2 treatment. n = 5 per group.

(C and D) 7G3-mediated inhibition of AML-9 engraftment (C) and homing efficiency (D) was attenuated in mice depleted of $CD122^{+}$ cells (+). n = 3 per group.

(E) Numbers of $CD34^{+}CD38^{-}$ AML-9 cells homed to the BM of irradiated NOD/SCID mice with (+) or without (–) $CD122^{+}$ cell depletion. n = 3 per group. Data are representative of results obtained with two AML samples. Error bars represent mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$ between selected groups.

weekly for 4 consecutive weeks at 0, 10, 30, and 100 mg/kg to a total of 32 cynomolgus monkeys (16 males and 16 females). CD123 binding by the chimeric variant was confirmed to be equivalent to the original parent 7G3 mouse mAb, and binding of both MAbs to cynomolgus CD123 was also demonstrated. There were no antibody-related effects on clinical observations nor on a comprehensive list of hematological parameters measured over 70 days after the first antibody treatment (data not shown). Overall, these data indicate that a CD123-targeting antibody does not exert adverse effects on normal hematopoiesis and are consistent with our NOD/SCID mouse experiments demonstrating that 7G3 treatment can specifically inhibit AML engraftment.

In Vivo Treatment with 7G3 Targets AML-LSCs

In order to determine if key properties of LSC such as self-renewal are targeted, serial transplantation was performed following in vivo 7G3 treatment. While 10 weeks of 7G3 treatment did not overtly decrease the engraftment of AML-10 in the BM or spleen of primary engrafted mice (Figure 5A), the AML cells harvested from 7G3-treated mice had significantly impaired homing ability to the BM and spleens of secondary recipient mice compared with IgG2a-treated controls (Figure 5B). The repopulation ability was also significantly impaired: while eight of nine secondary recipient mice transplanted with untreated control cells were engrafted, only three of eight mice inoculated with cells from 7G3-treated mice showed evidence of engraftment in the BM (Figure 5C). In the secondary mice, 7G3 also significantly reduced the proportion of $CD34^{+}CD38^{-}$ primitive cells in the BM (Figure 5D). Similar results were obtained in an independent experiment with AML-9 cells (Figure S4). In addition, when antibody treatment was combined with a suboptimal dose of cytarabine (Ara-C) to assess the potential for synergistic effects against another independent AML sample (AML-10), 7G3 again caused a marked reduction in the proportion of secondary mice engrafted (Figure S5). Collectively over three experiments, 26 of 27 (96%) secondary mice showed evidence of engraftment by cells harvested from IgG2a-treated mice, while only 12 of 23 (52%) were engrafted by cells from 7G3-treated mice. These results demonstrate that in vivo 7G3 administration specifically targets AML-LSCs in NOD/SCID mice, resulting in decreased homing and engraftment in secondary recipients.

7G3 Inhibits Spontaneous and IL-3-Induced Proliferation of Primitive AML Cells In Vitro

Due to the lack of cross-reactivity between the human and mouse IL-3 and CD123 systems, the ability of 7G3 to eliminate LSCs through targeting IL-3 signaling pathway is unable to be directly tested. To determine whether blocking IL-3 signaling can be one of the 7G3 inhibitory functions on AML-LSCs, we incubated different subtypes of primary AML cells with 7G3 or IgG2a in the medium containing IL-3. 7G3 inhibited exogenously added IL-3-induced proliferation in 32 of 35 primary AML samples (Figure 6A). Interestingly, 7G3 inhibited the growth of cells in nine of the samples to 50%–75% of control in the absence of exogenous IL-3, suggesting that these samples may possess an autocrine/paracrine IL-3 pathway or alternate growth mechanisms that can be blocked by 7G3. This profound

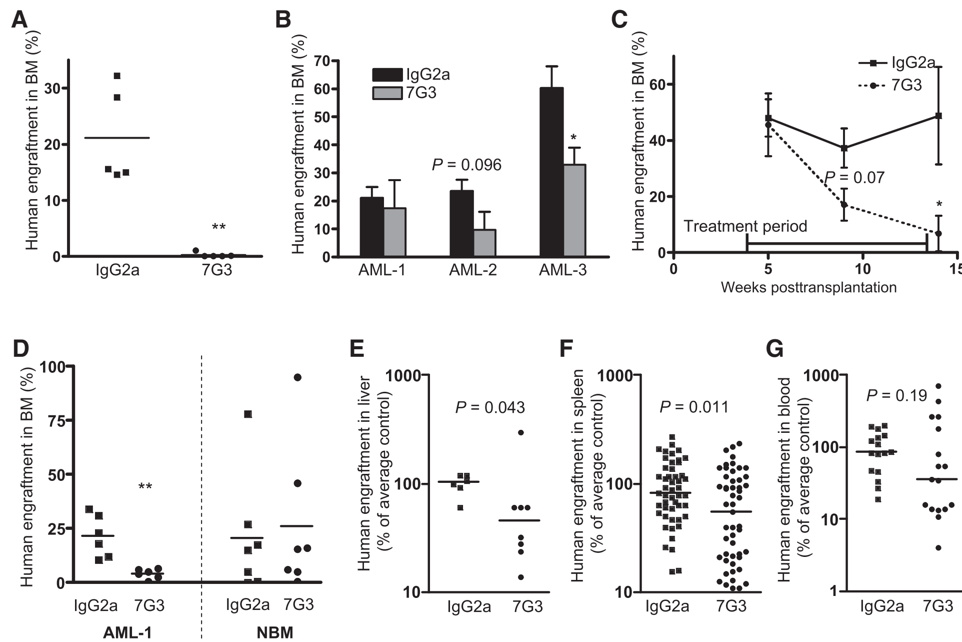

Figure 4. The Schedule of 7G3 Administration Influences Its Antileukemic Efficacy in NOD/SCID Mice

(A) Engraftment levels of AML-1 cells in the BM of mice treated with a single dose of IgG2a or 7G3 (300 μg) 6 hr prior to transplantation.

(B) Percentage of AML cells in the BM of mice (n = 5–6 per group) when the treatment was commenced 24 hr posttransplantation for four doses.

(C) Engraftment levels of AML-2 in the BM of mice when treatment was initiated at day 28 posttransplantation for 9 weeks' duration. n = 3–5 per group for each time point.

(D) Percentage of human AML-1 or NBM cells in the BM of mice after four doses of IgG2a or 7G3 starting on day 28 (AML-1) or 35 (NBM) posttransplantation.

(E–G) Assessment of leukemia infiltration in the liver (E), spleen (F), and peripheral blood (G) for the experiments in which initiating 7G3 treatments at 4 or 28 days did not cause a significant reduction in leukemic burden in the BM. Data are collected from six experiments for the different organs with n = 6–53 mice per group. Each symbol represents data from an individual mouse as a percent of average control for each experiment. Horizontal bars indicate the median in (E)–(G) and mean in (A) and (D). Otherwise results were expressed as mean ± SEM; *p < 0.05, **p < 0.01 between IgG2a and 7G3 groups.

inhibition by 7G3 was IL-3 specific since 7G3 had no effect on GM-CSF-induced cell proliferation (Figure 6B). In order to more directly link the 7G3-mediated reduction in proliferation to primitive AML cells, we demonstrated that 7G3 was able to significantly reduce IL-3-mediated survival of $CD34^+CD38^-CD123^+$ cells in three of four different patient samples (Figure 6C). In addition, 7G3 significantly reduced the survival of $CD34^+CD38^-$ cells from two samples (AML-14 and AML-15) in the absence of exogenously added IL-3. These data verify that 7G3 inhibits IL-3-induced and spontaneous proliferation of primary leukemic cells, and $CD34^+CD38^-CD123^+$ cell survival through binding to CD123.

7G3 Blocks IL-3-Mediated Signaling in AML Cells

We next tested whether 7G3 inhibited leukemic cell growth by blocking the activation of the IL-3R and its downstream pathway. The IL-3R β_c chain (CD131) was found to be coexpressed with CD123 on $CD34^+$ primary AML cells measured by both flow cytometry and PCR analyses (data not shown). Furthermore, IL-3-induced CD131 activation in primary AML cells and TF-1 assessed by tyrosine phosphorylation was inhibited by 7G3 in a concentration-dependent manner (Figure 6D and Figure S6, respectively). Inhibition of downstream STAT5 phosphorylation was also observed in TF1, bulk, and $CD34^+CD38^-$ AML cells (Figure 6E), as well as inhibition of both STAT5 and Akt phosphorylation in TF-1 cells (Figure S6), while the weakly neutralizing clones, 9F5 and 6H6 (Sun et al., 1996), were ineffective at inhibiting IL-3-mediated proliferation (data not shown) or signaling (Figure S6), demonstrating that different CD123 epitopes are functionally distinct. Collectively, these in vitro studies establish that 7G3 has the potential to also target LSCs by blocking IL-3-mediated signaling. Thus, in a clinical context, CD123 targeting has the potential to deliver antileukemic effects via activation of host immunity and inhibition of the IL-3 pathway.

DISCUSSION

In this report, we show that AML-LSCs can be targeted with the CD123-specific 7G3 mAb, resulting in impaired human AML cell engraftment and proliferation in NOD/SCID mice and improved long-term survival. The mechanism of LSC impairment by 7G3 treatment in the NOD/SCID model appeared complex and multifactorial, involving inhibition of LSC homing to the BM niche, and stimulation of residual innate immunity in NOD/SCID recipients. Although the consequences of blocking huIL-3 signaling with 7G3 in LSCs cannot be fully assessed in NOD/SCID mice, the in vitro data we generated showed marked impairment of the signaling, survival, and proliferation of primitive $CD34^+CD38^-$ AML cells. Since this subpopulation is highly enriched for LSCs, this result strongly suggests that impairment of IL-3 signaling will also be part of the multifactorial mechanism of action of 7G3 in a human context. Collectively, our results

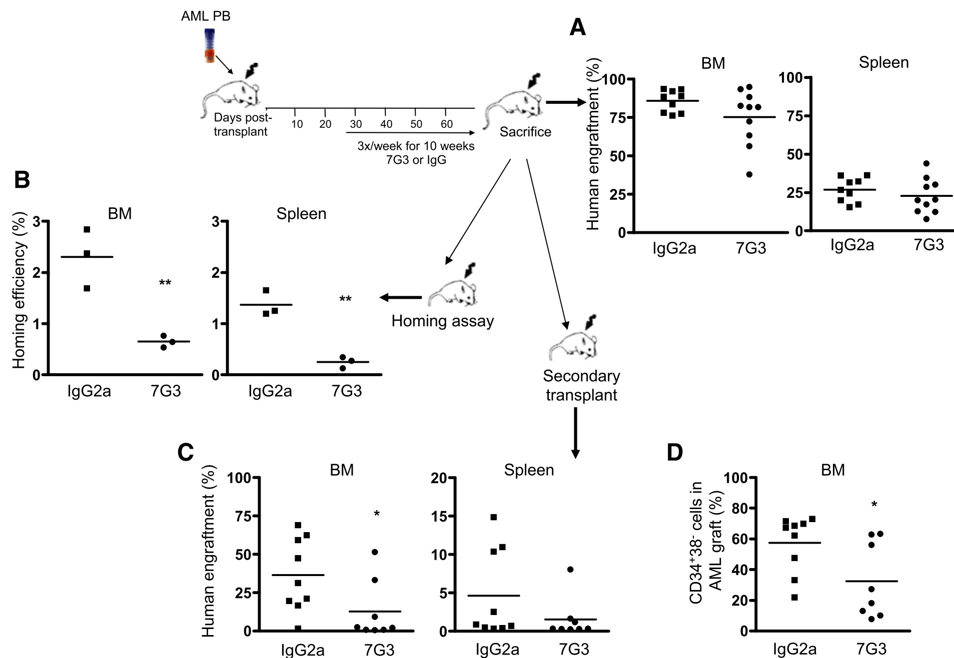

Figure 5. 7G3 Inhibits Self-Renewal Ability of AML-LSCs

(A) Engraftment levels of AML-10 cells in BM and spleen after 10 weeks of 7G3 or IgG2a treatment. The schedule of antibody treatment is shown in the schematic overview.

(B–D) (B) Homing efficiency, (C) levels of engraftment in the BM and spleen, and (D) the percentage of $CD34^{+}CD38^{-}$ cells in the BM of secondary recipient mice. Mice in (C) and (D) were analyzed at 12 weeks posttransplantation. Each symbol represents a single mouse, and horizontal bars indicate the mean value. *p < 0.05, **p < 0.01 between the two groups.

demonstrate that CD123 is an important marker for the targeting of LSCs and downstream progenitors that are capable of rapid proliferation. Our studies also show that, while the NOD/SCID mouse strain is immune deficient due to depleted T, B, and NK cells, it still proves to be an effective animal model for the preclinical testing of antibody-mediated immunotherapy.

Targeting LSCs by means of the 7G3 antibody against CD123 is an attractive approach, since (1) this receptor has been widely shown to be selectively overexpressed in LSCs; (2) the IL-3R classically stimulates multiple biological functions; and (3) 7G3 has the dual benefit of being a blocking antibody as well as mediating ADCC by effector cells providing additional and specific efficacy against leukemic cells, which a small molecule inhibitor of downstream signaling (e.g., JAK/STAT) may not be able to provide. Initial in vitro characterization showed that 7G3 robustly impaired IL-3 binding to its receptor in a broad panel of AML samples, thereby preventing IL-3-dependent CD131 tyrosine phosphorylation and downstream signaling, which are required to promote both cell survival and proliferation (Guthridge et al., 2000, 2006). Furthermore, inhibition of AML proliferation by 7G3 in the absence of exogenous IL-3 in 9 of 32 samples suggests that there is autocrine or paracrine secretion of IL-3 in some AML samples at physiologically significant levels. These experimental data are consistent with other reports demonstrating the expression of IL-3 mRNA and protein in primary AML samples (Guan et al., 2003; Nowak et al., 1999), as well as elevated serum IL-3 levels associated with leukemic burden in AML patients (Elbaz and Shaltout, 2001). By contrast, normal $CD34^{+}CD38^{-}$ cell proliferative potential is not affected by IL-3 (De Bruyn et al., 2000), and lineage-negative NBM cells did not have detectable IL-3 mRNA expression (Guan et al., 2003), suggesting that normal HSC function is relatively independent of IL-3 and is consistent with IL-3 being dispensable for normal hematopoiesis in IL-3-deficient mice (Nishinakamura et al., 1996). In AML, the level of CD123 expression and responsiveness to cytokines including IL-3 have been associated with poor prognosis (Graf et al., 2004; Testa et al., 2002, 2004; Tsuzuki et al., 1997). Thus, 7G3 inhibition of the CD123 signaling pathway in the context of AML patients, many of whom are likely to express high levels of circulating IL-3 (Elbaz and Shaltout, 2001), may provide significant additional benefit beyond the mechanisms we have already uncovered with the NOD/SCID model.

Residual murine NK cells, macrophages, or other host immune cells clearly contribute to the action of 7G3. Depletion of innate immunity in NOD/SCID mice with anti-CD122 mAb significantly, but not completely, attenuated the inhibitory effects of 7G3 on the homing and repopulating abilities of AML-LSCs. Additional evidence supporting this mechanism of 7G3-mediated inhibition of LSC function includes evidence that the Fc portion of 7G3 is critical for its activity, as well as the reduced potency of 7G3 in a NOD/SCID strain without NK cell activity. These findings support the further modification of 7G3 to enhance ADCC activity.

Our experiments provide two key findings that support the development of MAbs targeting CD123 as a novel therapy for AML. First, combined with those from other groups, our data showed that CD123 was highly expressed on the surface of $CD34^{+}CD38^{-}$ populations enriched for AML-LSCs compared

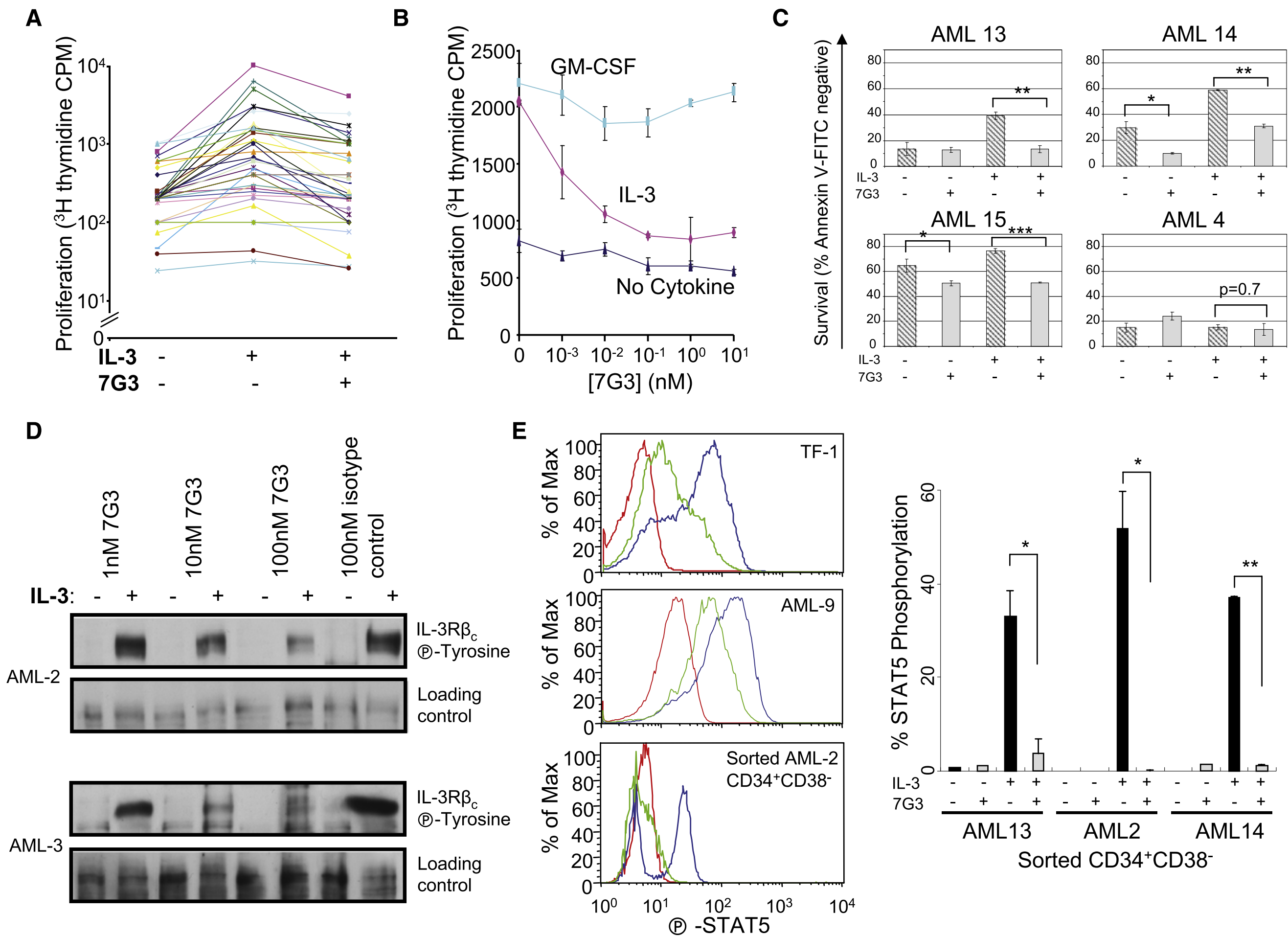

Figure 6. mAb 7G3 Inhibits Proliferation of Primary AML Cells

(A) Inhibition of primary AML cell proliferation by 7G3. Each line represents an individual AML sample exposed to the three different conditions. n = 35.

(B) Concentration-dependent effects of 7G3 on the proliferation of a primary AML sample induced by GM-CSF (0.1 ng/ml) or IL-3 (1 ng/ml). Data are representative of results obtained with 21 different AML samples.

(C) 7G3 inhibits IL-3-mediated survival of isolated $CD34^+CD38^-CD123^+$ primary AML cells. The percentage of surviving cells is shown.

(D) Western blot showing that 7G3 inhibits IL-3-induced CD131 tyrosine phosphorylation in a dose-dependent manner. n = 2 AML samples.

(E) 7G3 inhibits IL-3-induced phosphorylation of STAT5 in TF-1, primary AML, and sorted $CD34^+CD38^-$ AML cells shown by representative histograms of intracellular FACS (red, no IL-3; blue, IL-3 with IgG2a; green, IL-3 with 7G3); bar graphs represent the cumulative data in sorted $CD34^+CD38^-$ cells. Mean ± SEM in triplicates (B) or duplicates (C and E); $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p \leq 0.0001$ between indicated groups.

to their normal hematopoietic counterparts from both newborn CB and adult BM. Reduction of AML engraftment by ex vivo 7G3 treatment with less effect on normal HSCs, in comparison with the nonspecific ablation of both normal and AML sample engraftment by the antibody against HLA-A,B,C epitope, is consistent with the CD123 expression data. Similarly, in vivo 7G3 treatment appears to preferentially reduce AML engraftment relative to NBM xenograft. Second, the marked impairment of engraftment in secondary recipients demonstrates that 7G3 treatment targets the AML-LSCs in vivo, impairing LSC homing and reducing the repopulation of secondary recipients similar to the data from ex vivo treatment. Overall, this establishes 7G3 as a compelling LSC therapeutic in this preclinical model. Interestingly, the IF injection method established that at least a part of the action of 7G3 on LSC homing occurred during lodgment in microenvironmental niches and not during circulation through the blood, or during extravasation across endothelial membranes, since this method directly bypasses the latter processes by delivering cells to the femoral cavity.

The clinical potential for a CD123-targeting mAb is supported by three lines of evidence. First, our study has shown that ex vivo or in vivo 7G3 treatments selectively target AML cells compared with their normal counterparts. Second, toxicity testing in primates has shown that a chimeric IgG1 variant of 7G3 had no significant effects on any measured hematological parameters over 70 days. If normal hematopoiesis or HSCs had been affected, alteration of some of the parameters would be expected within this time frame. Third, the same chimeric variant mAb is being investigated in a phase I clinical trial as weekly treatment of patients with relapsed or refractory or high-risk AML. To date, with a total of >180 infusions administered to 26 patients comprising five dose-level cohorts up to 10 mg/kg, no signal of

treatment-related toxicity has been detected from hematology, biochemistry, or vital signs. Other than two mild infusion reactions, only one serious adverse event, an infection, was considered possibly related to treatment with the mAb. The incidence of adverse events did not increase with escalating dose, no grade 3–4 adverse events have been considered treatment related, and unrelated adverse events have been consistent with complications and risks of AML (A.W. Roberts et al., 2008, ASH Annual Meeting, abstract).

Although 7G3 effectively targeted AML-LSCs, it was most successful under conditions where the leukemic burden was low. When 7G3 treatment began at 4 weeks posttransplantation, BM engraftment was significantly impaired in only two of five primary AMLs. However, in this model of established AML, 7G3 distinctly reduced the AML burden in peripheral hematopoietic tissues (spleen, liver) in the majority of the samples we tested, perhaps due to a greater access of innate immune cells eliminating 7G3-coated AML cells (Fujii et al., 2007). While sample-to-sample variability was encountered at high leukemic burden, the increased effectiveness of 7G3 at low leukemic burden suggests a potential application of anti-CD123 treatment during remission following treatment with conventional chemotherapeutic agents.

The concept of antibody targeting of malignancy is well established. For example, several MAbs directed at hematological malignancies have been evaluated in clinical trials, including rituximab (which targets CD20) and epratuzumab (CD22) in B cell malignancies, alemtuzumab (CD52) in chronic lymphocytic leukemia, daclizumab (CD25) in T cell malignancies, and gemtuzumab ozogamicin (CD33) in AML. However, these mAb therapies are unlikely to target CSCs, and, while impressive cytoreduction and clinical responses have been observed, none are curative. Therefore, the multifaceted properties of 7G3 shown in this preclinical model of AML support a broader proposal for CSC-targeted cancer drug development (Wang, 2007) in which potential therapies that target key traits of CSCs are identified and tested using primary patient samples in relevant in vivo xenotransplantation models. The ongoing clinical evaluation of a chimeric CD123 mAb in advanced AML (http://clinicaltrials.gov/ct2/show/NCT00401739?term=CSL360&rank=1) will be the first of its kind to test whether the significant activity of an LSC-targeted mAb therapy in the xenograft models shown in this study translates into a clinical benefit for patients. Ultimately, this clinical testing will also provide more definitive proof of a role for IL-3 in the pathology of AML.

EXPERIMENTAL PROCEDURES

AML Patient Samples, Normal Hematopoietic Cells, and Cell Lines

Patient samples were collected after informed consent according to institutional guidelines, and studies were approved by the Royal Adelaide Hospital Human Ethics Committee, Melbourne Health Human Research Ethics Committee, Research Ethics Board of the University Health Network, and the South Eastern Sydney and Illawarra Area Health Service Human Research Ethics Committee. Diagnosis was made using cytomorphology, cytogenetics, and leukocyte antigen expression and evaluated according to the French-American-British (FAB) classification. Mononuclear cells were enriched by Lymphoprep (Axis-Shield PLC, Dundee, Scotland) or Ficoll (GE Healthcare, Uppsala, Sweden) density gradient separation and frozen in liquid nitrogen. Human CB and NBM cells were obtained from full-term deliveries or consenting patients receiving hip replacement surgery, or commercially from Cambrex Corporation (East Rutherford, NJ) and Lonza (Basel, Switzerland), respectively, and processed as previously described (Mazurier et al., 2003).

Ex Vivo Antibody Treatment

Thawed AML or normal hematopoietic cells were incubated with IgG2a, 7G3, 7G3 F(ab′)2, 9F5, 9F5 F(ab′)2, 6H6, or 6H6 F(ab′)2 (10 μg/ml) for 2 hr in X-VIVO 10 medium (Cambrex Corporation or Lonza) supplemented with 15%–20% bovine serum albumin-insulin-transferrin (StemCell Technologies Inc, Vancouver, Canada) at 37°C before i.v. transplantation into sublethally irradiated NOD/SCID mice for repopulating assays. Engraftment was measured at 4–10 weeks at two different time points.

Xenotransplantation of Human Cells into NOD/SCID Mice and In Vivo Antibody Treatment

Animal studies were performed under the institutional guidelines approved by the University Health Network/Princess Margaret Hospital Animal Care Committee or the Animal Care and Ethics Committee of the University of New South Wales. Transplantation of human cells into NOD/SCID mice was performed as previously described (Mazurier et al., 2003). Briefly, all mice received sublethal irradiation 24 hr before i.v. or IF transplantation with either 5–10 × 10^6 human AML cells, 3 × 10^5 lineage-depleted $CD34^+$ CB cells, 8 × 10^6 BM cells, or 1 × 10^6 sorted $CD34^+$ BM cells per mouse. Anti-CD122 antibody purified from the hybridoma cell line TM-β1 (generously provided by Professor T. Tanaka, Hyogo University of Health Sciences) (Tanaka et al., 1993) was injected intraperitoneally (i.p.) immediately after irradiation (200 μg/mouse). Engraftment levels of human AML and normal hematopoietic cells were evaluated by the percentage of $huCD45^+$ cells by flow cytometry (Lock et al., 2002). The number of $CD34^+CD38^-$ AML cells in the BM and spleen was also calculated based on the average number of cells harvested, and the engraftment levels and percent of $CD34^+CD38^-$ AML cells in each mouse. To measure effects on LSC activity, secondary transplantations were also performed by i.v. transplantation of 7–10 × 10^6 AML cells isolated from the BM (two femurs and two tibias) of IgG2a- or 7G3-treated primary mice into secondary recipient mice.

For in vivo testing, IgG2a or 7G3 (300 μg per injection) was injected i.p. into mice three times a week with schedules described in the legends to each figure. In vivo treatment of 7G3 was also tested in combination with the chemotherapeutic reagent Ara-C as described in Figure S5.

In Vivo Homing Assay

Homing assays were performed on ex vivo 7G3-treated cells, sorted $CD34^+CD38^-$ cells from primary patient samples, or cells harvested from previously engrafted mice, as previously described (Jin et al., 2006). Briefly, cells harvested from BM and spleen of mice transplanted 16 hr previously were stained with anti-human CD45-FITC, CD38-PE, and CD34-PC5 followed by flow cytometry for human cells using 5 × 10^4 to 5 × 10^6 collected events. Homing efficiency of human cells into the mouse tissues was calculated based on the number of total $huCD45^+$ cells in the tissue and the number of cells injected.

Cell Staining, Sorting, and Flow Cytometry

For flow cytometry, cells were stained as previously described (Bonnet and Dick, 1997; Lock et al., 2002) with conjugated anti-human antibodies against CD15, CD14, CD19, CD33, CD34, CD38, and CD45 (BD Biosciences, or BioLegend, CA). CD123 expression was measured with anti-CD123 clone 9F5, and RFI was determined by the ratio of the geometric mean of the 9F5-stained signal to matched isotype control. Stained cells were analyzed using FACScan or FACSCalibur flow cytometers (BD Biosciences). For sorting, cells were stained with anti-human antibodies against CD34, CD38, and CD123, and propidium iodide was also added to exclude dead cells when the cells were sorted using Moflo and BD Aria cell sorters (BD Biosciences).

Survival Analysis of $CD34^+CD38^-CD123^+$ AML Cells

Sorted cells plated at 1.5 × 10^5 cells/ml in IMDM/0.5% FCS were treated with 150 μg/ml 7G3 or IgG2a (clone BM4) for 30 min prior to addition of 1 nM IL-3. Cells were analyzed for survival at 48 and 72 hr by staining with 1:100 Annexin V-FLUOS (Roche, Basel, Switzerland) as described previously (Guthridge et al., 2006). Absolute cell number was also assessed by addition of 50 μl Flow-Count fluorospheres (Beckman Coulter).

Proliferation Assays

AML cell-growth responses to IL-3 or GM-CSF were measured by ^{3}H-thymidine assay as previously described (Lopez et al., 1988). Briefly, 2×10^4 mononuclear cells per well in 96-well plates were stimulated with IL-3 (1 ng/ml) or GM-CSF (0.1 ng/ml) in the presence of 0–10 nM 7G3 or IgG2a in 200 μl IMDM + 10% HI-FCS (Hyclone, UT) for 48 hr with 0.5 μCi of ^{3}H-thymidine (MP Biomedicals Australasia, Sydney, Australia) added for the last 6 hr of culture. Cells were deposited onto glass fiber paper using a Packard Filtermate cell harvester (PerkinElmer Life and Analytical Sciences, Melbourne, Australia) and counted using a Top Count (PerkinElmer). All cytokines were supplied by R&D Systems (MN).

Cytokine Signaling Assays

Phosphorylation of signaling proteins was detected by immunoprecipitation and immunoblots. TF-1 and primary AML cells were washed and rendered quiescent overnight before incubation with IgG2a, 9F5, 6H6, or 7G3 (0–100 nM) for 20 min on ice. Cells were then stimulated with 1 nM IL-3 for 10 min at 37°C. Cells were lysed in NP-40 lysis buffer, and CD131 was immunoprecipitated using 1C1 and 8E4 antibodies conjugated to Sepharose beads (Guthridge et al., 2004). Immunoprecipitates were subjected to SDS-PAGE and immunoblotting as previously described (Guthridge et al., 2004). Antibodies used were the following: antiphosphotyrosine mAb 4G10 (Upstate Biotechnology Inc, NY), anti-phospho-Akt Ser473 (Cell Signaling Technology Inc, MA), and, anti-phosphorylated STAT5 mAb (Zymed Laboratories Inc, CA). All antibodies were used according to manufacturers' instructions. Blots were stripped and reprobed with antibody to βc (1C1) as a loading control.

For intracellular FACS, quiescent TF-1, bulk, and sorted primary AML cells were stimulated with 20 ng/ml IL-3 plus 20 μg/ml IgG2a or 7G3 for 1 hr. Sorted subpopulations were incubated with 150 μg/ml 7G3 or IgG2a for 30 min on ice before stimulation with 1 nM IL-3 for 15 min. Cells were fixed with BD Cytofix Buffer (BD Biosciences), methanol permeabilized, and stained with anti-phosphoSTAT5 (BD Biosciences) or isotype control. Cells were then analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Statistical Analysis

Data are presented as the mean ± SEM. The significance of differences between groups was determined using the unpaired, two-sided Student's t test, or the nonparametric Mann-Whitney U test. Survival curves were compared using the log rank test.

SUPPLEMENTAL DATA

Supplemental Data include six figures and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00207-0.

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