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CD47 Is an Adverse Prognostic Factor and Therapeutic Antibody Target on Human Acute Myeloid Leukemia Stem Cells

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SUMMARY

Acute myeloid leukemia (AML) is organized as a cellular hierarchy initiated and maintained by a subset of self-renewing leukemia stem cells (LSC). We hypothesized that increased CD47 expression on human AML LSC contributes to pathogenesis by inhibiting their phagocytosis through the interaction of CD47 with an inhibitory receptor on phagocytes. We found that CD47 was more highly expressed on AML LSC than their normal counterparts, and that increased *CD47* expression predicted worse overall survival in three independent cohorts of adult AML patients. Furthermore, blocking monoclonal antibodies directed against CD47 preferentially enabled phagocytosis of AML LSC and inhibited their engraftment in vivo. Finally, treatment of human AML LSC-engrafted mice with anti-CD47 antibody depleted AML and targeted AML LSC. In summary, increased CD47 expression is an independent, poor prognostic factor that can be targeted on human AML stem cells with blocking monoclonal antibodies capable of enabling phagocytosis of LSC.

INTRODUCTION

According to the cancer stem cell model, tumors are organized as a cellular hierarchy maintained by a small pool of self-renewing cancer stem cells that must be eliminated in order to eradicate the tumor (Jordan et al., 2006; Reya et al., 2001). For the development of cancer stem cell-targeted therapies, it is necessary to identify molecules and pathways that are preferentially expressed in these cancer stem cells and that are critical for pathogenesis.

To date, human acute myeloid leukemia (AML) stem cells (LSC) are the most well studied cancer stem cell population (Wang and Dick, 2005). AML is an aggressive malignancy with 5 year overall survival between 30%–40%, and much lower for those over age 65 (Estey and Dohner, 2006; Lowenberg et al., 1999). Cytogenetic abnormalities are prognostic in adults with AML; however, up to 50% have a normal karyotype (Byrd et al., 2002; Grimwade et al., 1998). In these patients, the presence of specific molecular mutations can provide prognostic information, particularly internal tandem duplications within the *fms*-related tyrosine kinase 3 gene (*FLT3*-ITD) (Mrozek et al., 2007; Schlenk et al., 2008).

In published reports assaying a variety of subtypes of AML, LSC were found to be negative for expression of lineage markers (Lin^-), positive for expression of CD34, and negative for expression of CD38 (Bonnet and Dick, 1997; Wang and Dick, 2005). We have recently shown that the $Lin^-CD34^+CD38^-CD90^-$ fraction of human cord blood contains a non-hematopoietic stem cell (HSC) multipotent progenitor (MPP) and have hypothesized that this MPP is the cell of origin for human AML (Majeti et al., 2007). Consistent with this hypothesis, we have shown that pre-leukemic mutations occur in a clonal HSC population, eventually leading to the development of LSC at the MPP stage in AML or the granulocyte-macrophage progenitor (GMP) stage in myeloid blast crisis chronic myeloid leukemia (CML) (Jamieson et al., 2004; Miyamoto et al., 2000; Weissman, 2005).

We report here the identification of higher expression of CD47 on AML LSC compared to their normal counterparts, HSC and MPP, a finding corroborated by microarray gene expression analysis (Majeti et al., 2009). CD47 is a widely expressed transmembrane protein (Brown and Frazier, 2001). CD47 serves as the ligand for signal regulatory protein alpha (SIRPα), which is expressed on phagocytic cells including macrophages and dendritic cells, that when activated initiates a signal transduction cascade resulting in inhibition of phagocytosis (Barclay and Brown, 2006; Blazar et al., 2001; Okazawa et al., 2005;

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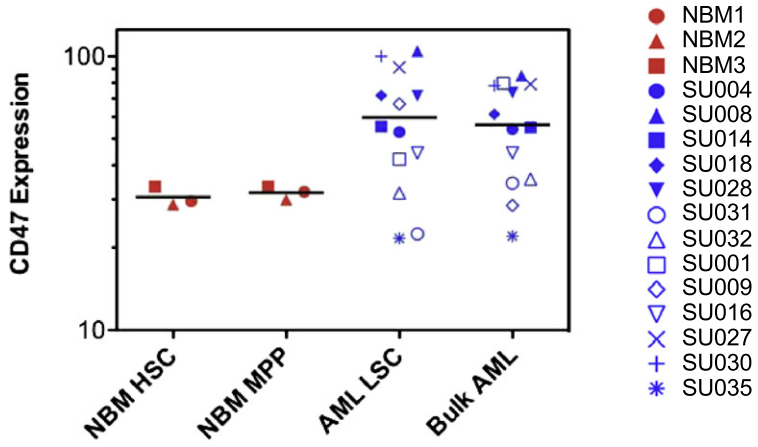

B

Sample	Age	Gender	Primary/ Secondary	De Novo/ Relapsed	Cytogenetics	Flt-3 ITD	% CD34	WHO Classification	FAB
SU001	59	Female	Primary	Relapsed	Normal	Negative	99	AML not-otherwise specified	M2
SU004	47	Female	Primary	Relapsed	Normal	Positive	38	AML not-otherwise specified	M5
SU008	64	Male	Primary	De Novo	Normal	Positive	3	AML not-otherwise specified	M1
SU009	43	Male	Primary	De Novo	Normal	Negative	73	AML with multilineage dysplasia without antecedent MDS	M2
SU014	59	Male	Primary	De Novo	Normal	Positive	18	AML not-otherwise specified	NS
SU016	59	Male	Primary	De Novo	Normal	Positive	10	AML with multilineage dysplasia without antecedent MDS	NS
SU018	21	Male	Primary	Relapsed	Normal	Positive	55	AML not-otherwise specified	M5
SU027	61	Male	Primary	Relapsed	Failed to grow	Unknown	10	AML not-otherwise specified	M5
SU028	53	Female	Primary	Relapsed	Complex	Positive	50	AML not-otherwise specified	M5
SU030	53	Female	Primary	De Novo	Normal	Positive	40	AML with multilineage dysplasia without antecedent MDS	M4
SU031	31	Female	Primary	De Novo	inv (16)(p13;q22)	Negative	97	AML with inv(16)(p13;q22) or t(16;16)(p13;q22)	M4eo
SU032	47	Male	Primary	De Novo	Normal	Negative	68	AML not-otherwise specified	M5
SU035	48	Male	Primary	De Novo	Failed to grow	Negative	98	AML not-otherwise specified	M5

Figure 1. CD47 Is More Highly Expressed on AML LSC Compared to Their Normal Counterparts

(A) Relative CD47 expression on normal bone marrow HSC ($Lin^-CD34^+CD38^-CD90^+$) and MPP ($Lin^-CD34^+CD38^-CD90^-CD45RA^-$), as well as LSC ($Lin^-CD34^+CD38^-CD90^-$) and bulk leukemia cells from human AML samples, was determined by flow cytometry. Mean fluorescence intensity was normalized for cell size and against lineage-positive cells to account for analysis on different days. The same sample of normal bone marrow (red, n = 3) or AML (blue, n = 13) is indicated by the same symbol in the different populations. Normalized mean expression (and range) for each population were as follows: HSC 30.6 (28.8–33.4), MPP 31.8 (30.0–33.4), LSC 59.8 (21.6–104.7), and bulk AML 56.3 (22.1–85.1). The differences between the mean expression of HSC with LSC ($p = 0.003$), HSC with bulk leukemia ($p = 0.001$), MPP with LSC ($p = 0.004$), and MPP with bulk leukemia ($p = 0.002$) were statistically significant using a two-sided Student's t test. The difference between the mean expression of AML LSC compared to bulk AML was not statistically significant with $p = 0.50$ using a paired two-sided Student's t test.

(B) Clinical and molecular characteristics of primary human AML samples manipulated in vitro and/or in vivo.

Oldenborg et al., 2000, 2001). In our own studies, we have found that expression of mouse CD47 in a human AML cell line inhibits phagocytosis and facilitates engraftment in immunodeficient mice, and that CD47 expression on mouse HSC and progenitors increases upon mobilization and is required for engraftment upon transplantation (Jaiswal et al., 2009 [this issue of *Cell*]). We hypothesize that increased expression of CD47 on human AML contributes to pathogenesis by inhibiting phagocytosis of these cells through the interaction of CD47 with SIRPα.

RESULTS

CD47 Is More Highly Expressed on AML LSC Than on Their Normal Counterparts and Is Associated with the FLT3-ITD Mutation

In our investigation of several mouse models of myeloid leukemia, we identified increased expression of CD47 on mouse leukemia cells compared to normal bone marrow (Jaiswal et al., 2009). This prompted investigation of CD47 expression on human AML LSC and their normal counterparts. Using flow cytometry, CD47 was more highly expressed on multiple specimens of AML LSC than on normal bone marrow HSC and MPP (Figure 1). This increased expression extended to the bulk leukemia cells, which expressed CD47 similarly to the LSC-enriched fraction.

Examination of a subset of these samples indicated that CD47 surface expression correlated with *CD47* mRNA expression (Figure S1 available online). To investigate CD47 expression across morphologic, cytogenetic, and molecular subgroups of AML, gene expression data from a previously described cohort of 285 adult patients were analyzed (Valk et al., 2004). No significant difference in *CD47* expression among FAB (French-American-British) subtypes was found (Figure S2A). In most cytogenetic subgroups, *CD47* was expressed at similar levels, except for cases harboring t(8;21)(q22;q22), a favorable risk group that

had a statistically significant lower *CD47* expression (Figure S2B). In molecularly characterized AML subgroups, no significant association was found between *CD47* expression and mutations in the tyrosine kinase domain of *FLT3* (*FLT3*-TKD), overexpression of *EVI1*, or mutations in *CEBPA*, *NRAS*, or *KRAS*. However, higher *CD47* expression was strongly correlated with the presence of *FLT3*-ITD ($p < 0.001$), which is observed in nearly one-third of AML with normal karyotypes and is associated with worse overall survival (Mrozek et al., 2007; Schlenk et al., 2008). This finding was separately confirmed in two independent datasets of 214 and 137 AML patients (Table S1) (Bullinger et al., 2008; Jongen-Lavrencic et al., 2008).

Identification and Separation of Normal HSC from Leukemia Cells in the Same Patient Based on Differential CD47 Expression

In the LSC-enriched $Lin^-CD34^+CD38^-$ fraction of specimen SU008, a rare population of $CD47^{lo}$-expressing cells was detected, in addition to the majority $CD47^{hi}$-expressing cells (Figure 2A). These populations were isolated by fluorescence-activated cell sorting (FACS) to >98% purity and either transplanted into newborn NOG mice or plated into complete methylcellulose. The $CD47^{hi}$ cells failed to engraft in vivo or form any colonies in vitro, as can be observed with some AML specimens (Ailles et al., 1997). However, the $CD47^{lo}$ cells engrafted with normal myelo-lymphoid hematopoiesis in vivo and formed numerous morphologically normal myeloid colonies in vitro (Figures 2B and 2C). This specimen harbored the *FLT3*-ITD mutation, which was detected in the bulk leukemia cells (Figure 2D). The purified $CD47^{hi}$ cells contained the *FLT3*-ITD mutation and therefore were part of the leukemic clone, whereas the $CD47^{lo}$ cells did not. Human cells isolated from mice engrafted with the $CD47^{lo}$ cells contained only wild-type *FLT3*, indicating that the $CD47^{lo}$ cells contained normal hematopoietic progenitors.

Increased CD47 Expression in Human AML Is Associated with Poor Clinical Outcomes

We hypothesized that increased CD47 expression on human AML contributes to pathogenesis and predicted that AML with higher expression of CD47 would be associated with worse clinical outcomes. Consistent with this hypothesis, analysis of a previously described group of 285 adult AML patients with diverse cytogenetic and molecular abnormalities (Valk et al., 2004) revealed that a dichotomous stratification of patients into low CD47 and high CD47 expression groups was associated with a significantly increased risk of death in the high expressing group ($p = 0.03$, Figures S3A–S3C). The association of overall survival with this dichotomous stratification of *CD47* expression was validated in a second test cohort of 242 adult patients (Metzeler et al., 2008) with normal karyotypes (NK-AML) ($p = 0.01$, Figures S3A and S3D).

Applying this stratification to a distinct validation cohort of 137 adult patients with normal karyotypes (Bullinger et al., 2008), we confirmed the prognostic value of *CD47* expression for both overall and event-free survival (Figure 3). Analysis of clinical characteristics of the low and high CD47 expression groups in this cross-validation cohort also identified statistically significant differences in white blood cell (WBC) count and *FLT3*-ITD status and no differences in rates of complete remission and type of consolidative therapy including allogeneic transplantation (Table S1). Kaplan-Meier analysis demonstrated that high *CD47* expression at diagnosis was significantly associated with worse event-free and overall survival (Figures 3A and 3B). Patients in the low CD47 expression group had a median event-free survival of 17.1 months compared to 6.8 months in the high CD47 expression group, corresponding to a hazard ratio of 1.94 (95% confidence interval 1.30 to 3.77, $p = 0.004$). For overall survival, patients in the low CD47 expression group had a median of 22.1 months compared to 9.1 months in the high CD47 expression group, corresponding to a hazard ratio of 2.02 (95% confidence interval 1.37 to 4.03, $p = 0.002$). When *CD47* expression was considered as a continuous variable, increased expression was also associated with a worse event-free ($p = 0.02$) and overall survival ($p = 0.02$).

Despite the association with *FLT3*-ITD (Table S1), increased *CD47* expression at diagnosis was significantly associated with worse event-free and overall survival in the subgroup of 74 patients without *FLT3*-ITD, when considered either as a binary classification (Figures 3C and 3D) or as a continuous variable ($p = 0.02$ for both event-free and overall survival). In multivariable analysis considering age, *FLT3*-ITD status, and *CD47* expression as a continuous variable, increased *CD47* expression remained associated with worse event-free survival with a hazard ratio of 1.33 (95% confidence interval 1.03 to 1.73, $p = 0.03$) and overall survival with a hazard ratio of 1.31 (95% confidence interval 1.00 to 1.71, $p = 0.05$) (Table S2).

Monoclonal Antibodies Directed against Human CD47 Preferentially Enable Phagocytosis of AML LSC by Human Macrophages

We hypothesized that increased CD47 expression on human AML contributes to pathogenesis by inhibiting phagocytosis of leukemia cells, leading us to predict that disruption of the CD47-SIRPα interaction with a monoclonal antibody directed against CD47 will preferentially enable the phagocytosis of AML LSC. Several anti-human CD47 monoclonal antibodies have been generated including some capable of blocking the CD47-SIRPα interaction (B6H12.2 and BRIC126) and others unable to do so (2D3) (Subramanian et al., 2006). The ability of these antibodies to enable phagocytosis of AML LSC, or normal human bone marrow $CD34^+$ cells, by human macrophages in vitro was tested. Incubation of AML LSC with human macrophages in the presence of IgG1 isotype control antibody or mouse anti-human CD45 IgG1 monoclonal antibody did not result in significant phagocytosis, as determined by either immunofluorescence microscopy (Figure 4A) or flow cytometry (Figure S5). However, addition of the blocking anti-CD47 antibodies B6H12.2 and BRIC126, but not the nonblocking anti-CD47 antibody 2D3, enabled phagocytosis of AML LSC (Figures 4A and 4C). No phagocytosis of normal $CD34^+$ cells was observed with any of the antibodies (Figure 4C).

Monoclonal Antibodies Directed against Human CD47 or Mouse SIRPα Enable Phagocytosis of AML LSC by Mouse Macrophages

The CD47-SIRPα interaction has been implicated as a critical regulator of xenotransplantation rejection in several cross-species

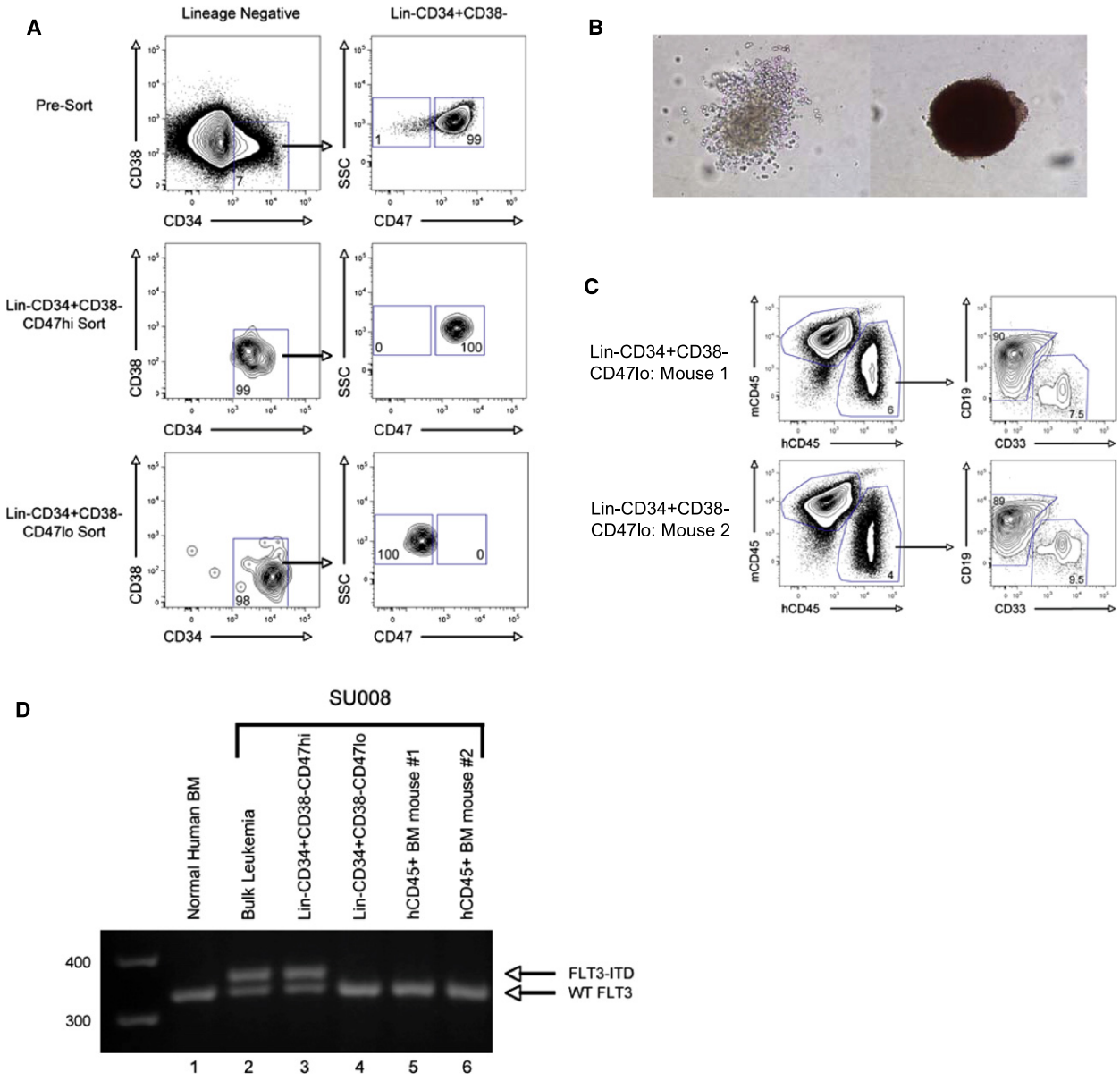

Figure 2. Identification and Separation of Normal HSC From Leukemia Cells in the Same Patient Based on Differential CD47 Expression

(A) CD47 expression on the Lin$^-$CD34$^+$CD38$^-$ LSC-enriched fraction of specimen SU008 was determined by flow cytometry. CD47hi- and CD47lo-expressing cells were identified and purified using FACS. The left panels are gated on lineage-negative cells, while the right panels are gated on Lin$^-$CD34$^+$CD38$^-$ cells.

(B) Lin$^-$CD34$^+$CD38$^-$CD47lo and Lin$^-$CD34$^+$CD38$^-$CD47hi cells were plated into complete methylcellulose, capable of supporting the growth of all myeloid colonies. Fourteen days later, myeloid colony formation was determined by morphologic assessment. Representative CFU-G/M (left) and BFU-E (right) are presented.

(C) Lin$^-$CD34$^+$CD38$^-$CD47lo cells were transplanted into two newborn NOG mice. Twelve weeks later, the mice were sacrificed and the bone marrow was analyzed by flow cytometry for the presence of human CD45$^+$CD33$^+$ myeloid cells and human CD45$^+$CD19$^+$ lymphoid cells.

(D) Normal bone marrow HSC, bulk SU008 leukemia cells, Lin$^-$CD34$^+$CD38$^-$CD47hi cells, Lin$^-$CD34$^+$CD38$^-$CD47lo cells, or human CD45+ cells purified from the bone marrow of mice engrafted with Lin$^-$CD34$^+$CD38$^-$CD47lo cells were assessed by PCR for the presence of the *FLT3*-ITD mutation. The wild-type (WT) *FLT3* and the *FLT3*-ITD products are indicated.

transplants; however, there are conflicting reports of the ability of CD47 from one species to bind and stimulate SIRPα of a different species (Ide et al., 2007; Subramanian et al., 2006; Takenaka et al., 2007). In order to directly assess the effect of inhibiting the interaction of human CD47 with mouse SIRPα, the in vitro phagocytosis assays described above were conducted with mouse macrophages. Incubation of AML LSC with mouse macrophages in the presence of IgG1 isotype control antibody or mouse anti-human CD45 IgG1 monoclonal antibody did not result in significant phagocytosis, as determined by either immunofluorescence microscopy (Figure 4B) or flow cytometry (Figure S5). However, addition of the blocking anti-CD47 antibodies

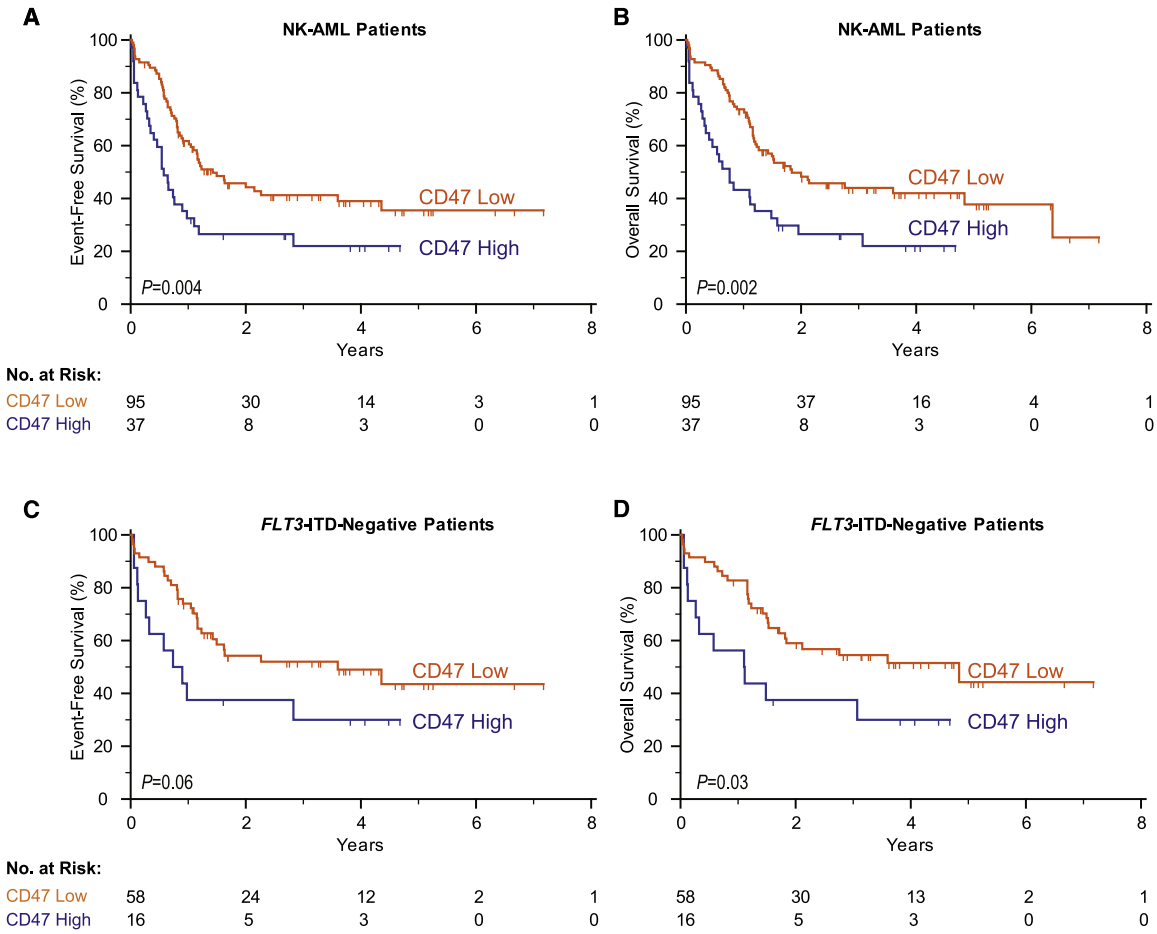

Figure 3. Increased CD47 Expression in Human AML Is Associated with Poor Clinical Outcomes

Event-free (A and C) and overall (B and D) survival of 132 AML patients with normal cytogenetics (A and B) and the subset of 74 patients without the *FLT3*-ITD mutation (C and D). Patients were stratified into low CD47 and high CD47 expression groups based on an optimal threshold (28% high, 72% low) determined by microarray analysis from an independent training data set. The significance measures are based on log-likelihood estimates of the p value, when treating the model with *CD47* expression as a binary classification.

B6H12.2 and BRIC126, but not the nonblocking 2D3, enabled phagocytosis of AML LSC (Figures 4B and 4C). The CD47-SIRPα interaction was alternatively disrupted by a monoclonal antibody directed against mouse SIRPα, which also enabled phagocytosis of AML LSC (Figure 4C).

A Monoclonal Antibody Directed against CD47 Does Not Induce Apoptosis of AML LSC

Antibodies directed against CD47 have been shown to directly induce apoptosis of several malignant hematopoietic cell lines, as well as primary human chronic lymphocytic leukemia B cells, only when immobilized or crosslinked (Kikuchi et al., 2004, 2005; Mateo et al., 1999; Uno et al., 2007). These prior reports raise the alternative hypothesis that anti-CD47 antibodies induce apoptosis of AML LSC, which are then recognized by macrophages and phagocytosed. In order to assess the ability of the blocking anti-CD47 antibody B6H12.2 to directly induce apoptosis of primary human AML LSC, these cells were incubated in vitro with antibodies as described above, but in the absence of macrophages, and expression of Annexin V was determined by flow cytometry. No increase in Annexin V-positive apoptotic cells was detected with the anti-CD47 antibody compared to controls over the time period tested (Figure 4D). Even when plate-bound, the anti-CD47 antibody did not induce apoptosis of AML LSC (Figure S7D). Furthermore, phagocytosis of AML LSC was detected as early as 15 min after incubation with blocking anti-CD47 antibody, while no apoptosis was detected at 2 hr (Figure S7E). These results indicate that the blocking anti-CD47 antibody B6H12.2 does not directly induce apoptosis of human AML LSC.

A Monoclonal Antibody Directed against Human CD47 Inhibits AML LSC Engraftment and Depletes AML In Vivo

The ability of the blocking anti-CD47 antibody B6H12.2 to target AML LSC in vivo was tested. First, a pre-coating strategy was utilized in which AML LSC were purified by FACS and incubated with IgG1 isotype control, anti-human CD45, or anti-human CD47 antibody. An aliquot of the cells was analyzed for coating

by staining with a secondary antibody, demonstrating that both anti-CD45 and anti-CD47 antibody bound the cells (Figure S9A). The remaining cells were transplanted into newborn NOG mice that were analyzed for leukemic engraftment 13 weeks later. In all but one mouse, the isotype control and anti-CD45 antibody-coated cells exhibited long-term leukemic engraftment; however, most mice transplanted with cells coated with anti-CD47 antibody had no detectable leukemia engraftment (Figure S9B).

Next, a treatment strategy was utilized in which mice were first engrafted with human AML LSC and then administered daily intraperitoneal injections of 100 μg of either mouse IgG or anti-CD47 antibody for 14 days, with leukemic engraftment determined pre- and post-treatment. Analysis of the peripheral blood showed near complete elimination of circulating leukemia in mice treated with anti-CD47 antibody, often after a single dose, with no response in control mice (Figures 5A and 5B). Similarly, there was a significant reduction in leukemic engraftment in the bone marrow of mice treated with anti-CD47 antibody, while leukemic involvement increased in control IgG-treated mice (Figures 5C, 5D, and S10A). Histologic analysis of the bone marrow identified monomorphic leukemic blasts in control IgG-treated mice (Figure 5E, panels 1 and 2) and cleared hypocellular areas in anti-CD47 antibody-treated mice (Figure 5E, panels 4 and 5). In the bone marrow of some anti-CD47 antibody-treated mice that contained residual leukemia, macrophages were detected containing phagocytosed pyknotic cells (Figure 5E, panels 3 and 6).

A Monoclonal Antibody Directed against Mouse CD47 Enables Phagocytosis of Mouse AML and Does Not Deplete Normal HSC In Vivo

CD47 is expressed at low levels on most normal tissues, including HSC. In order to investigate the viability of targeting CD47 as a therapeutic strategy, we utilized a mouse model of AML and a blocking anti-mouse CD47 monoclonal antibody (MIAP301) (Oldenborg et al., 2001). A serially transplantable mouse model of AML was generated by transduction of 5-fluouracil-treated wild-type bone marrow with a retrovirus encoding HoxA9 and Meis1, as well as GFP (Lessard and Sauvageau, 2003). These mouse leukemia cells exhibited a 3- to 5-fold increase in CD47 surface expression compared to normal bone marrow (data not shown), similar to that observed with human AML. We first investigated the ability of the blocking anti-mouse CD47 monoclonal antibody to enable phagocytosis of the mouse leukemia cells and found that unlike an isotype-matched control, anti-mouse CD47 antibody enabled phagocytosis of GFP-positive leukemia cells by mouse macrophages in vitro (Figures 6A and 6B). Next, wild-type mice were administered daily intraperitoneal injections of 200 μg of anti-mouse CD47 antibody for 14 days. This dose resulted in antibody coating of 100% of total bone marrow cells (data not shown). The mice appeared grossly normal and were sacrificed at the end of the treatment course. Analysis of the bone marrow showed no difference in overall cellularity (data not shown), percentage of $Lin^{-}Kit^{+}Sca^{+}$ (KLS) cells (Figure 6C), or percentage of HSC (Figure 6D). Complete blood counts showed no evidence of anemia but did indicate isolated neutropenia in the anti-CD47 antibody-treated mice (Table S3). Metabolic panels showed no serological evidence of hepatic or renal damage (Table S4). Finally, in a pilot experiment, we found that treatment of mouse leukemia-engrafted mice with the anti-mouse CD47 antibody resulted in a statistically significant increased survival compared to control IgG (Figure S11). These results suggest that targeting CD47 with a blocking monoclonal antibody yields no unacceptable toxicity and is a viable therapeutic strategy.

A Monoclonal Antibody Directed against Human CD47 Enables Phagocytosis of AML In Vivo and Targets AML LSC

The in vivo mechanism of the anti-human CD47 antibody was investigated using two approaches to determine if the blocking B6H12.2 anti-CD47 antibody eliminates human AML in vivo by enabling phagocytosis of these cells. First, primary human AML LSC were transduced with a lentivirus expressing GFP and transplanted into NOG mice. Engrafted mice were treated with a single dose of mouse IgG or anti-CD47 antibody, and 4 hr later bone marrow, spleen, and liver were examined by flow cytometry for the presence of GFP-positive human leukemia cells within F4/80-positive mouse phagocytes. Unlike IgG control-treated mice, human GFP^{+} AML cells were detected within phagocytes from all three tissues in anti-CD47-treated mice (Figures 7A and 7B). In the second experiment, mouse phagocytes were depleted in human AML LSC-engrafted mice prior to treatment with anti-CD47 antibody by administering liposomal clodronate, which accumulates in lysosomes resulting in death of phagocytes (Figure S12A). Depletion of phagocytes inhibited the ability of anti-CD47 antibody to eliminate human AML from both the peripheral blood and bone marrow in vivo (Figure 7C).

Finally, in vivo targeting of AML LSC was investigated. First, the percentage of $CD34^{+}$ LSC-enriched human leukemia cells present in the bone marrow after treatment was determined by flow cytometry. Treatment with anti-CD47 antibody resulted in a statistically significant decrease in the percentage of human $CD34^{+}$ leukemia cells remaining in the bone marrow after treatment (Figures 7D and S10B). Next, targeting of AML LSC was functionally assessed by secondary transplantation of bone marrow from IgG control or anti-CD47 antibody-treated mice. Secondary mice transplanted from IgG-treated mice engrafted human leukemia in the peripheral blood (Figure S13) and bone marrow (Figure 7E). However, secondary mice transplanted from anti-CD47-treated mice developed no engraftment in the blood or marrow, which could be the result of in vivo antibody coating with anti-CD47. Regardless, the lack of secondary engraftment clearly indicates that treatment with anti-CD47 antibody targeted AML LSC in vivo.

DISCUSSION

We report here the identification of higher expression of CD47 on AML LSC compared to their normal counterparts and hypothesize that increased expression of CD47 on human AML contributes to pathogenesis by inhibiting phagocytosis of these cells through the interaction of CD47 with SIRPα (Figure S14A). Consistent with this hypothesis, we demonstrate that increased expression of CD47 in human AML is associated with decreased

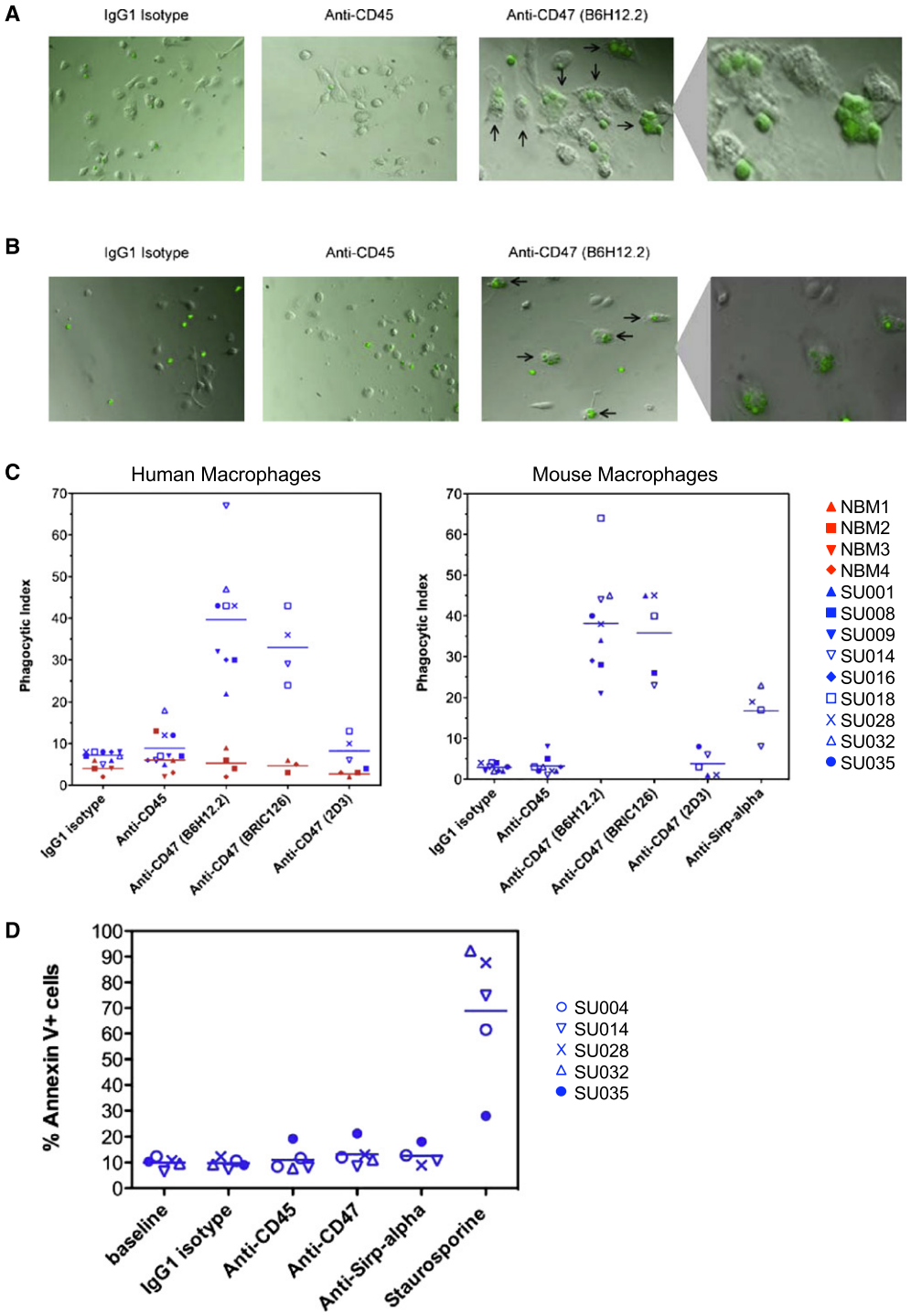

Figure 4. Monoclonal Antibodies Directed against Human CD47 Preferentially Enable Phagocytosis of Human AML LSC by Human and Mouse Macrophages In Vitro

(A and B) CFSE-labeled AML LSC were incubated with human peripheral blood-derived macrophages (A) or mouse bone marrow-derived macrophages (B) in the presence of IgG1 isotype control, anti-CD45 IgG1, or anti-CD47 (B6H12.2) IgG1 antibody. These cells were assessed by immunofluorescence microscopy for the presence of fluorescently labeled LSC within the macrophages (indicated by arrows).

(C) CFSE-labeled AML LSC or normal bone marrow $CD34^+$ cells were incubated with human (left) or mouse (right) macrophages in the presence of the indicated antibodies and then assessed for phagocytosis by immunofluorescence microscopy. The phagocytic index was determined for each condition by calculating the number of ingested cells per 100 macrophages. For AML LSC, the differences between isotype or anti-CD45 antibody with blocking anti-CD47 antibody treatment (B6H12.2 and BRIC126) were statistically significant with $p < 0.001$ for all pairwise comparisons with human and mouse macrophages. For human

overall survival. We also demonstrate that disruption of the CD47-SIRPα interaction with monoclonal antibodies directed against CD47 preferentially enables phagocytosis of AML LSC by macrophages, inhibits engraftment, and targets AML LSC in vivo. Together, these results establish the rationale for considering the use of an anti-CD47 monoclonal antibody as a therapy for human AML.

The enabling of phagocytosis by blocking monoclonal antibodies directed against CD47 is a mechanism of action for a therapeutic monoclonal antibody in the treatment of cancer that has not, to our knowledge, been previously described. Currently approved antibody therapies are believed to act via stimulation of antibody-dependent cellular cytotoxicity (ADCC), via disruption of critical receptor-ligand interactions, or through unknown mechanisms (Adams and Weiner, 2005). Blocking anti-CD47 monoclonal antibodies would treat human AML by enabling phagocytosis and elimination of AML LSC (Figure S14B). In support of this mechanism of action in vivo, we show that treatment of human AML LSC-engrafted mice with anti-CD47 antibody results in rapid phagocytosis of AML cells (Figures 7A and 7B), and that depletion of phagocytes with clodronate abrogates this effect (Figure 7C).

As demonstrated here, CD47 contributes to pathogenesis by conferring a survival advantage to LSC and progeny blasts through evasion of phagocytosis by the innate immune system. Moreover, some dendritic cells express SIRPα (Braun et al., 2006; Latour et al., 2001; Sarfati et al., 2008; Seiffert et al., 1999), and we propose that increased CD47 expression on AML LSC also serves to prevent the activation of adaptive T cell immune responses.

AML LSC are enriched in the $Lin^-CD34^+CD38^-$ fraction, which in normal bone marrow contains HSC and MPP. The identification of cell-surface molecules that can distinguish between leukemic and normal stem cells is essential for flow cytometry-based assessment of minimal residual disease (MRD) and for the development of prospective separation strategies for use in cellular therapies. Several candidate molecules, including CD123 (Jordan et al., 2000), CD44 (Jin et al., 2006), CD96 (Hosen et al., 2007), CLL-1 (van Rhenen et al., 2007), and now CD47, have recently been identified. We demonstrate not only that CD47 is more highly expressed on AML LSC compared to normal HSC and MPP but also that this differential expression can be used to separate normal HSC/MPP from leukemia cells. This demonstration of the prospective separation of normal HSC from leukemia cells in the same patient sample offers the possibility of leukemia-depleted autologous HSC transplantation therapies.

Targeting of CD47 on AML LSC with Therapeutic Monoclonal Antibodies

Cell-surface molecules preferentially expressed on AML LSC compared to their normal counterparts are candidates for targeting with therapeutic monoclonal antibodies. Thus far, several molecules, including CD33 (Adams and Weiner, 2005), CD44 (Jin et al., 2006), CD123 (Jin et al., 2009), and now CD47, have been targeted on AML. Here we report that a monoclonal antibody directed against CD47 targets AML LSC in vivo, as shown by direct reduction in the percentage of human $CD34^+$ LSC-enriched leukemia cells in the bone marrow and complete elimination of engraftment in secondary transplants (Figures 7D and 7E).

Targeting of leukemia cells and cell lines with anti-CD47 antibodies has previously been reported to directly induce apoptosis. Treatment of primary human B-CLL cells was shown to induce caspase-independent cell death (Mateo et al., 1999), while a different anti-CD47 antibody was shown to induce apoptosis of several hematopoietic cell lines (Kikuchi et al., 2004, 2005; Uno et al., 2007). These reports raise the alternative hypothesis that anti-CD47 antibodies induce apoptosis of AML LSC, which are then recognized by macrophages and phagocytosed. However, several caveats must be considered when comparing these prior reports to our current study. First, the report on B-CLL involved a mature lymphocytic neoplasm, which is very biologically different from immature aggressive AML, and demonstrated apoptosis not with soluble antibody but only with crosslinking of antibody, which can result in different effects. Second, the additional reports utilized cell lines and not primary leukemia cells, which are very biologically distinct regarding both proliferation and cell death. Ultimately, we feel that it is not possible to extrapolate the effect of anti-CD47 antibodies from these reports to primary human AML cells.

Several lines of evidence suggest that targeting of CD47 with a monoclonal antibody acts by disrupting the CD47-SIRPα interaction, thereby preventing a phagocytic inhibitory signal, rather than by acting through induction of apoptosis, ADCC, or other mechanisms. First, two blocking anti-CD47 antibodies enabled AML LSC phagocytosis, while one nonblocking antibody did not, even though all three bind the cells similarly (Figures 4C and S6). Second, an anti-mouse SIRPα antibody also enabled phagocytosis of human AML LSC by mouse macrophages, demonstrating phagocytosis without direct binding of antibody to AML LSC (Figure 4C). Third, in the case of the B6H12.2 antibody used for most of our experiments, no direct induction of apoptosis of primary AML LSC was detected when added either as a soluble antibody or as an immobilized plate-bound antibody (Figures 4D and S7D). Fourth, phagocytosis of AML LSC was detected as early as 15 min after addition of anti-CD47 antibody, while no apoptosis was detected at 2 hr (Figures 4D and S7E). In fact, only minimal Annexin V-positive staining was detected on Jurkat cells 2 hr after incubation with immobilized plate-bound anti-CD47 antibody (Figure S7C). Fifth, if phagocytosis were occurring secondary to apoptosis, then depletion of phagocytes with clodronate should not inhibit the effect of the antibody, which would still directly kill the leukemia cells. However, clodronate did inhibit the ability of anti-CD47 antibody to deplete

macrophages, the differences between AML LSC and normal $CD34^+$ cells were statistically significant for B6H12.2 ($p < 0.001$) and BRIC126 ($p = 0.002$). For mouse macrophages, the difference between isotype control and anti-SIRPα antibody was statistically significant ($p = 0.02$).

(D) AML LSC were incubated in the presence of the indicated antibodies or the staurosporine-positive control as described above, but in the absence of macrophages. At the end of the incubation, apoptotic cells were identified by Annexin V staining as determined by flow cytometry. No statistically significant increase in apoptosis was detected with any of the antibodies.

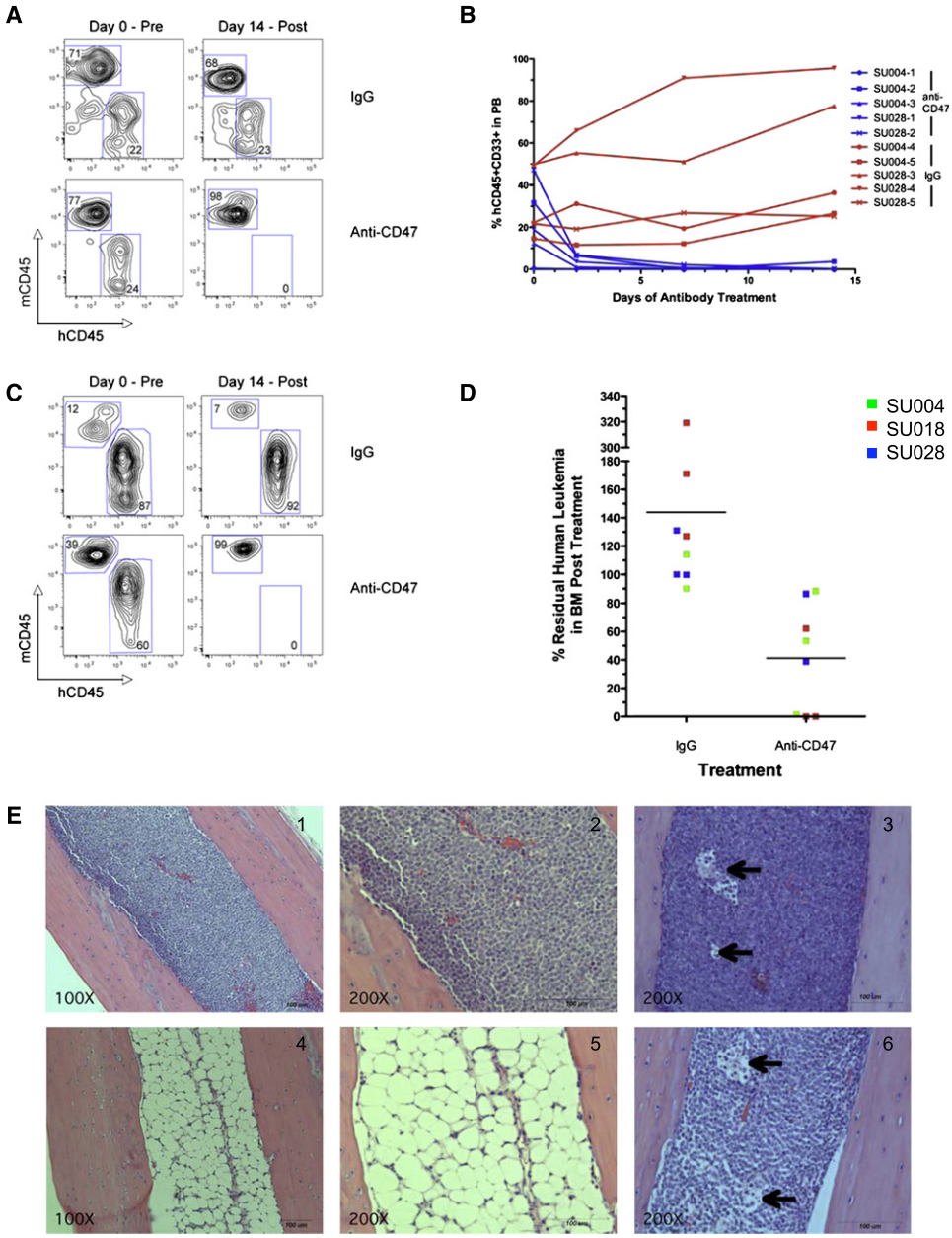

Figure 5. A Monoclonal Antibody Directed against Human CD47 Depletes AML In Vivo

(A–D) Newborn NOG mice were transplanted with AML LSC, and 8–12 weeks later, peripheral blood (A and B) and bone marrow (C and D) were analyzed for baseline engraftment prior to treatment with anti-CD47 (B6H12.2) or control IgG antibody (Day 0). Mice were treated with daily 100 μg intraperitoneal injections for 14 days, at the end of which they were sacrificed and peripheral blood and bone marrow were analyzed for the percentage of human $CD45^{+}CD33^{+}$ leukemia. (A) Pre- and post-treatment human leukemic chimerism in the peripheral blood from representative anti-CD47 antibody and control IgG-treated mice as determined by flow cytometry. (B) Summary of human leukemic chimerism in the peripheral blood assessed on multiple days during the course of treatment demonstrated elimination of leukemia in anti-CD47 antibody-treated mice compared to control IgG treatment ($p = 0.007$). (C) Pre- and post-treatment human leukemic chimerism in the bone marrow from representative anti-CD47 antibody or control IgG-treated mice as determined by flow cytometry. (D) Summary of human leukemic chimerism in the bone marrow on day 14 relative to day 0 demonstrated a dramatic reduction in leukemic burden in anti-CD47 antibody-treated mice compared to control IgG treatment ($p = 0.006$).

(E) H&E sections of representative mouse bone marrow cavities from mice engrafted with SU004 AML LSC post-treatment with either control IgG (panels 1 and 2) or anti-CD47 antibody (panels 4 and 5). IgG-treated marrows were packed with monomorphic leukemic blasts, while anti-CD47-treated marrows were hypocellular, demonstrating elimination of the human leukemia. In some anti-CD47 antibody-treated mice that contained residual leukemia, macrophages were detected containing phagocytosed pyknotic cells (panels 3 and 6, arrows).

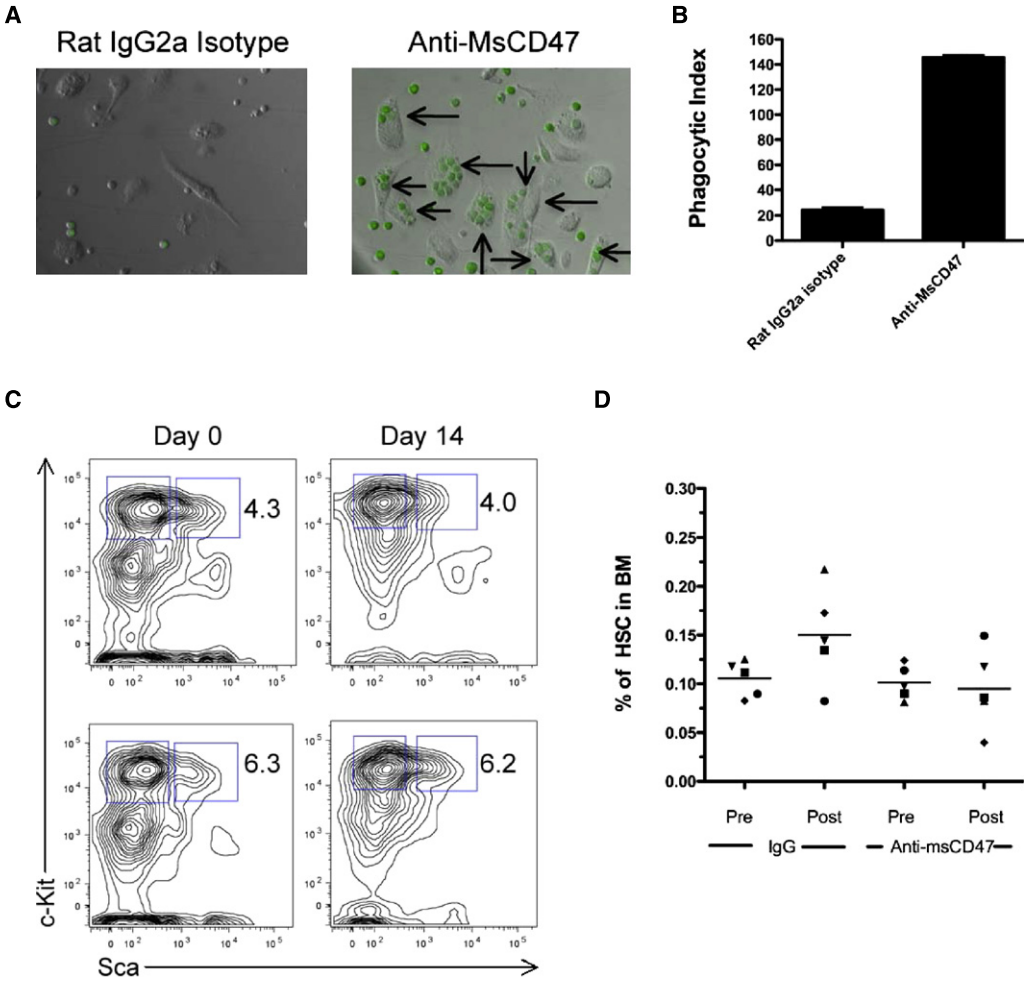

Figure 6. A Monoclonal Antibody Directed against Mouse CD47 Enables Phagocytosis of Mouse AML and Does Not Deplete Normal HSC In Vivo

(A) GFP$^+$ mouse AML cells were incubated with mouse bone marrow-derived macrophages in vitro in the presence of 10 μg/ml of rat IgG2a isotype control or anti-mouse CD47 antibody for 2 hours. Phagocytosis of GFP$^+$ leukemia cells was observed by fluorescence microscopy (arrows).

(B) Quantitative analysis of phagocytosis was determined by calculating the phagocytic index in triplicate assays. Anti-MsCD47 antibody enabled a statistically significant increase in phagocytosis of mouse leukemia cells compared to isotype control ($p < 0.001$). Error bars indicate the standard deviation of triplicate measurements.

(C and D) C57BL/6 wild-type mice were treated for 14 days with daily 200 μg intraperitoneal injections of either anti-msCD47 or rat IgG control antibody. Bone marrow from these mice was aspirated pre- and post-treatment and indicated no effect of either treatment on the frequency of Lin$^-$Kit$^+$Sca$^+$ (KLS) cells (C) or Lin$^-$Kit$^+$Sca$^+$Flk2$^-$CD34$^-$ HSC (D) in the bone marrow. Representative flow cytometry plots are shown in (C). No differences in the percentage of HSC pre- and post-treatment were observed with either control IgG ($p = 0.09$) or anti-msCD47 ($p = 0.81$).

human AML (Figure 7C), indicating that enabling of phagocytosis is the most likely mechanism. Finally, the isotype-matched anti-CD45 antibody, which also binds LSC, failed to produce the same effects, making ADCC less likely (Figure 4). In fact, the B6H12.2 antibody is mouse isotype IgG1, which is less effective at engaging mouse Fc receptors than antibodies of isotype IgG2a or IgG2b (Nimmerjahn and Ravetch, 2007). For human clinical therapies, blocking CD47 on AML LSC with humanized monoclonal antibodies should promote LSC phagocytosis through a similar mechanism, as indicated by the human macrophage-mediated in vitro phagocytosis (Figures 4A and 4C).

Higher CD47 expression is detected on AML LSC; however, CD47 is expressed on normal tissues, including bone marrow HSC. We identified a preferential effect of anti-CD47 antibodies in enabling the phagocytosis of AML LSC compared to normal bone marrow CD34$^+$ cells by human macrophages in vitro. In fact, no increased phagocytosis of normal CD34$^+$ cells compared to isotype control was detected, suggesting that blocking CD47 with monoclonal antibodies is a viable therapeutic strategy for human AML. We speculate that this difference is due to the presence of as yet unknown stimuli for phagocytosis on AML LSC that are lacking on normal CD34$^+$ cells. We have now administered a blocking anti-mouse CD47 antibody to normal

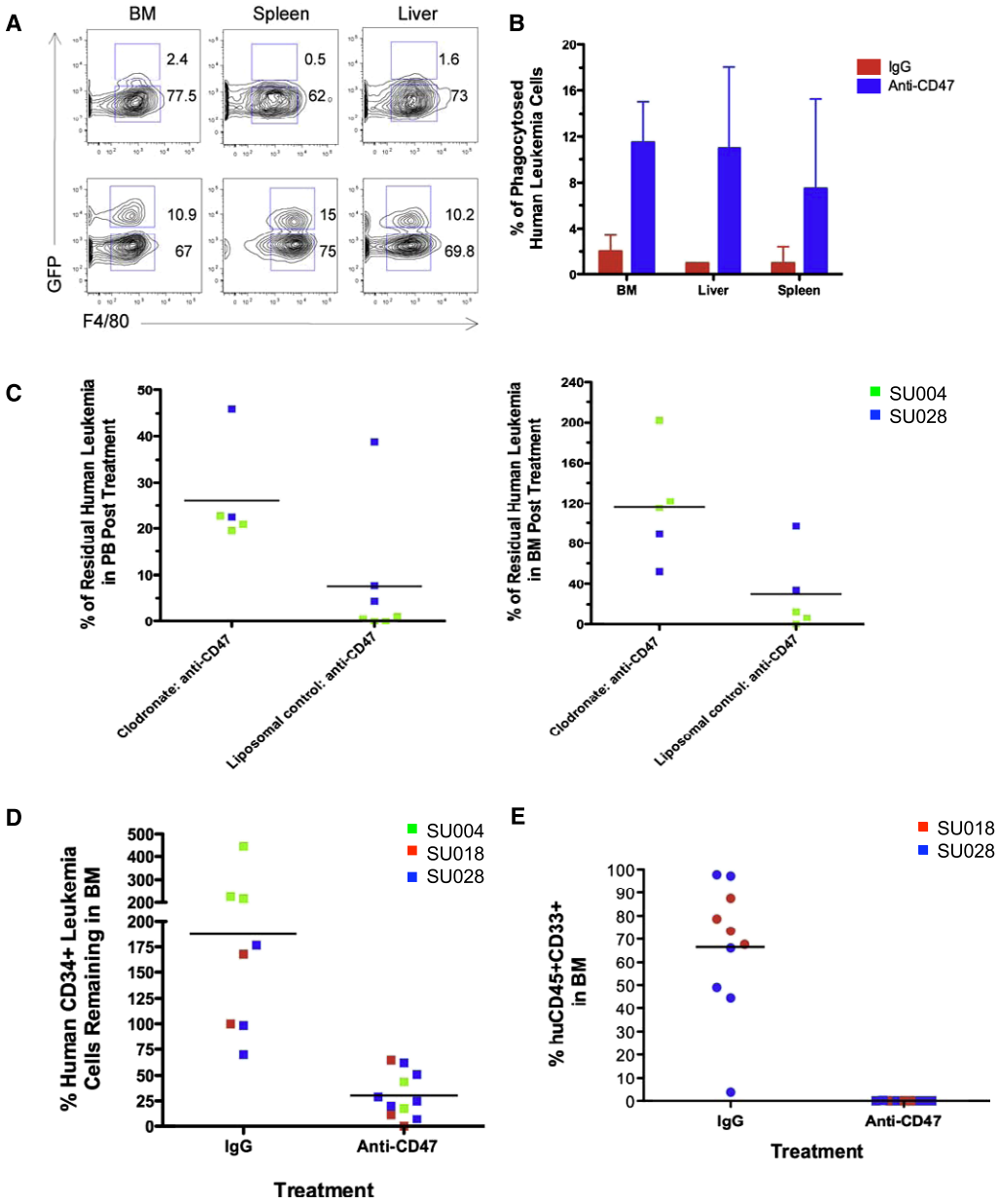

Figure 7. A Monoclonal Antibody Directed against Human CD47 Enables Phagocytosis of AML In Vivo and Targets LSC

(A and B) Flow cytometry plots (A) and quantitation (B) from NOG mice engrafted with lentivirally transduced GFP-positive SU028 AML LSC, 4 hr after treatment with a single 100 μg intraperitoneal dose of anti-CD47 antibody (B6H12.2) or control IgG (n = 2 for each). Cell suspensions from bone marrow, spleen, and liver were stained for human CD45 and mouse F4/80, which recognizes phagocytes. All plots are gated on human CD45-negative cells. Double-positive events represent GFP-positive leukemia cells within mouse phagocytes. Error bars indicate the standard deviation of duplicate measurements.

(C) NOG mice engrafted with the indicated AML LSC were treated with liposomal clodronate to deplete phagocytes and administered daily intraperitoneal injections of anti-CD47 antibody for 14 days. The percentage of residual human leukemia cells in the peripheral blood (left) and bone marrow (right) was determined as described above. Depletion of phagocytes resulted in a statistically significant inhibition of the ability of anti-CD47 antibody to eliminate human AML from both the peripheral blood ($p = 0.03$) and bone marrow ($p = 0.04$). Clodronate treatment by itself had no effect on leukemic engraftment (Figures S12A and S12B).

(D) The percentage of human $CD34^+$ LSC-enriched human leukemia cells remaining in the bone marrow after treatment with either IgG control or anti-CD47 antibody was determined by flow cytometry. Treatment with anti-CD47 antibody resulted in a statistically significant decrease ($p < 0.001$) compared to the control.

(E) 500,000 whole bone marrow cells from IgG control (n = 12) or anti-CD47 (n = 9) antibody-treated mice were secondarily transplanted into NOG mice. Twelve weeks later, secondary mice were sacrificed and analyzed for human leukemia engraftment in the peripheral blood (Figure S13) and bone marrow as described above. Secondary mice transplanted from IgG-treated mice engrafted human leukemia in the bone marrow, while secondary mice transplanted from anti-CD47-treated mice developed no engraftment ($p < 0.001$). Statistical significance was determined using Fisher's exact test.

mice on a schedule similar to the anti-human CD47 antibody and find coating of all bone marrow cells with no reduction in HSC, no liver or kidney toxicity, and only isolated neutropenia in complete blood counts (Figure 6 and Tables S3 and S4), suggesting that this antibody has no unacceptable toxicity and does not deplete normal mouse HSC.

The experimental evidence presented here provides the rationale for anti-CD47 monoclonal antibodies as monotherapy for AML. However, such antibodies may be equally, if not more effective as part of a combination strategy. The combination of a blocking anti-CD47 antibody with a second antibody able to bind an LSC-specific molecule (for example CD96) and engage Fc receptors on phagocytes may result in a synergistic stimulus for phagocytosis and specific elimination of AML LSC (Figure S14C). Furthermore, combinations of monoclonal antibodies to AML LSC that include blocking anti-CD47 and human IgG1 antibodies directed against two other cell-surface antigens will be more likely to eliminate leukemia cells with pre-existing epitope variants or antigen loss that are likely to recur in patients treated with a single antibody.

EXPERIMENTAL PROCEDURES

Human Samples

Normal human bone marrow mononuclear cells were purchased from AllCells Inc. (Emeryville, CA, USA). Human AML samples (Figure 1B) were obtained from patients at the Stanford Medical Center with informed consent, according to an IRB-approved protocol (Stanford IRB# 76935 and 6453). Human CD34-positive cells were enriched with magnetic beads (Miltenyi Biotech, Auburn, CA, USA).

Flow Cytometry Analysis and Cell Sorting

A panel of antibodies was used for analysis and sorting of AML LSC ($Lin^-CD34^+CD38^-CD90^-$, where lineage included CD3, CD19, and CD20), HSC ($Lin^-CD34^+CD38^-CD90^+$), and MPP ($Lin^-CD34^+CD38^-CD90^-CD45RA^-$) as previously described (Majeti et al., 2007). Analysis of CD47 expression was performed with an anti-human CD47 PE antibody (clone B6H12, BD Biosciences, San Jose CA, USA). For analysis of mouse bone marrow, the following antibodies were used: Sca1 PB, cKit Alexa 750, Flk2 PE, CD34 FITC, Lineage (CD3, CD4, CD5, CD8, B220, Mac1) PeCy5 (Ebiosciences, San Diego, CA, USA).

Anti-Human and -Mouse CD47 and Anti-Mouse SIRPα Antibodies

Monoclonal mouse anti-human CD47 antibodies included the following: BRIC126, IgG2b (Abcam, Cambridge, MA, USA), 2D3, IgG1 (Ebiosciences), and B6H12.2, IgG1. Monoclonal rat anti-mouse CD47 antibody used was MIAP301, IgG2a. The B6H12.2 and MIAP301 hybridomas were obtained from the American Type Culture Collection (Rockville, MD, USA). Antibody was either purified from hybridoma supernatant using protein G affinity chromatography according to standard procedures or obtained from BioXCell (Lebanon, NH, USA). Monoclonal rat anti-mouse SIRPα, P84, IgG1 was purchased from BD PharMingen (San Jose, CA, USA). Isotype controls included mouse IgG1 and rat IgG2a antibodies (Ebiosciences).

In Vitro Phagocytosis Assays

Human AML LSC or normal bone marrow $CD34^+$ cells were CFSE-labeled and incubated with either mouse or human macrophages in the presence of 7 μg/ml IgG1 isotype control, anti-CD45 IgG1, anti-CD47 (clones B6H12.2, BRIC126, or 2D3), or anti-mouse SIRPα antibody for 2 hr. Mouse GFP-positive leukemia cells were incubated with mouse macrophages in the presence of 10 μg/ml of rat IgG2a isotype control or anti-mouse CD47 (MIAP301) for 2 hr. Cells were then analyzed by fluorescence microscopy to determine the phagocytic index (number of cells ingested per 100 macrophages). In some experiments, cells were then harvested and stained with either a mouse or human macrophage marker and phagocytosed cells were identified by flow cytometry as $macrophage^+CFSE^+$. Statistical analysis using Student's t test was performed with GraphPad Prism. See Supplemental Experimental Procedures for detailed procedures.

In Vivo Antibody Treatment of Human AML LSC Engrafted Mice

1–2.5 × 10^5 FACS-purified LSC were transplanted into NOG pups. Eight to twelve weeks later, human AML engraftment ($hCD45^+CD33^+$ cells) was assessed in the peripheral blood and bone marrow by tail bleed and aspiration of the femur, respectively. Engrafted mice were then treated with daily intraperitoneal injections of 100 μg of anti-CD47 antibody or IgG control for 14 days. On day 15 mice were sacrificed and the peripheral blood and bone marrow were analyzed for AML.

In Vivo Human AML Phagocytosis Assay

A GFP encoding lentivirus was prepared from the pCDH-CMV construct (System Biosciences, Mountain View, CA, USA) using standard techniques. AML LSC from sample SU028 were transduced overnight and transplanted into newborn NOG pups as described. Twelve weeks later human $CD45^+CD33^+GFP^+$ leukemia engraftment was assessed in the peripheral blood, and GFP^+ human leukemia-engrafted mice were injected intraperitoneally with a single 100 μg dose of either anti-CD47 antibody (clone B6H12.2) or IgG control. Four hours later, mice were sacrificed and bone marrow, spleen, and liver were analyzed by flow cytometry for the presence of GFP^+ leukemia cells within F4/80-positive mouse phagocytes. The presence of human $CD45^-GFP^+$ mouse $F4/80^+$ events identified mouse phagocytes with ingested human leukemia cells.

In Vivo Macrophage Depletion

Liposomal clodronate and control liposomes were prepared as described (Jaiswal et al., 2009). Macrophages were depleted in AML LSC-engrafted NOG mice with the following treatment schedule: 200 μl of either clodronate or liposomal control was injected intravenously via the retro-orbital sinus 2 days prior to treatment of these mice with anti-CD47 antibody for IgG control. One hundred microliters of either clodronate or liposomal control was then injected in the same manner on days 2, 6, and 10 after initiation of daily antibody treatment. Mice were then sacrificed on day 14 to assess human leukemic engraftment as described.

Secondary Transplantation

AML LSC-engrafted mice treated with daily injections of either IgG control or anti-CD47 antibody were sacrificed at the end of 14 days of treatment. 5 × 10^5 whole bone marrow cells were transplanted into newborn NOG mice. Twelve weeks later peripheral blood and bone marrow was harvested and analyzed for human $CD45^+CD33^+$ leukemia engraftment as described.

AML Patients, Microarray Gene Expression Data, and Statistical Analysis

Gene expression and clinical data were analyzed for three previously described cohorts of adult AML patients: (1) a training dataset of 285 patients with diverse cytogenetic and molecular abnormalities described by Valk et al. (2004), (2) a test dataset of 242 patients with normal karyotypes described by Metzeler et al. (2008), and (3) a validation dataset of 137 patients with normal karyotypes described by Bullinger et al. (2008). See Supplemental Experimental Procedures for details of therapy. The clinical end points analyzed included overall and event-free survival, with events defined as the interval between study enrollment and removal from the study owing to a lack of complete remission, relapse, or death from any cause, with data censored for patients who did not have an event at the last follow-up visit. See Supplemental Experimental Procedures for detailed procedures.

SUPPLEMENTAL DATA

Supplemental Data include fourteen figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00650-3.

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