

CD47 Is Upregulated on Circulating Hematopoietic Stem Cells and Leukemia Cells to Avoid Phagocytosis

Siddhartha Jaiswal,^{1,*} Catriona H.M. Jamieson,² Wendy W. Pang,¹ Christopher Y. Park,¹ Mark P. Chao,¹ Ravindra Majeti,¹ David Traver,³ Nico van Rooijen,⁴ and Irving L. Weissman^{1,*}

¹Ludwig Center at Stanford, Stanford Cancer Center, Department of Pathology, and Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA

²Stem Cell Research Program, Moores UCSD Cancer Center

³Section of Cell and Developmental Biology

University of California San Diego, La Jolla, CA, 92093, USA

⁴Department of Molecular Cell Biology, Vrije Universiteit, VUMC, Amsterdam, The Netherlands

*Correspondence: sjaiswal@stanford.edu (S.J.), irv@stanford.edu (I.L.W.)

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SUMMARY

Macrophages clear pathogens and damaged or aged cells from the blood stream via phagocytosis. Cell-surface CD47 interacts with its receptor on macrophages, SIRP α , to inhibit phagocytosis of normal, healthy cells. We find that mobilizing cytokines and inflammatory stimuli cause CD47 to be transiently upregulated on mouse hematopoietic stem cells (HSCs) and progenitors just prior to and during their migratory phase, and that the level of CD47 on these cells determines the probability that they are engulfed *in vivo*. CD47 is also constitutively upregulated on mouse and human myeloid leukemias, and overexpression of CD47 on a myeloid leukemia line increases its pathogenicity by allowing it to evade phagocytosis. We conclude that CD47 upregulation is an important mechanism that provides protection to normal HSCs during inflammation-mediated mobilization, and that leukemic progenitors co-opt this ability in order to evade macrophage killing.

INTRODUCTION

Hematopoietic stem cells (HSCs) have the ability to migrate to ectopic niches in fetal and adult life via the blood stream (Christensen et al., 2004; Cumano and Godin, 2007; Morrison et al., 1995; Wright et al., 2001b). Once in the blood, HSCs home to VCAM-1⁺ endothelia using integrin $\alpha 4\beta 1$ (Wagers et al., 2002), then navigate the vascular beds of the marrow, spleen, and liver before returning to potential niches that secrete SDF-1, for which HSCs have responsive CXCR4 chemokine receptors (Peled et al., 1999; Wright et al., 2002). Macrophages lining vascular sinusoids at these sites function to remove damaged cells and foreign particles from the blood stream, sensing altered surface molecules. During inflammatory responses, macrophages become more phagocytically active in order to more effectively clear offending

pathogens (Wright et al., 1989). At these times, host cells are at increased risk of clearance and newly arriving stem cells might require additional protection against phagocytosis.

Like normal HSCs, leukemic stem and progenitor cells also have an intrinsic ability to migrate via circulation to ectopic marrow sites. By analogy with the normal HSCs, the leukemic stem cells (LSCs) must also be able to navigate through macrophage-lined vasculature of spleen, liver, and marrow as they travel to ectopic niches.

CD47 is an immunoglobulin-like protein that is known to interact functionally with integrins (Brown and Frazier, 2001) and thrombospondin-1 (Gao et al., 1996; Liu et al., 2001). It has been implicated in functions as diverse as neutrophil migration (Lindberg et al., 1996), axon extension (Miyashita et al., 2004), and T cell costimulation (Reinhold et al., 1997). In addition, CD47 is capable of interacting with its receptor SIRP α (Jiang et al., 1999) on macrophages to negatively regulate phagocytosis (Brown and Frazier, 2001). Lack of autonomous CD47 expression results in phagocytosis of red blood cells (Oldenberg et al., 2000), as well as T cells and whole bone marrow cells in a transplant setting (Blazar et al., 2001). Thus, CD47 functions as a “don’t eat me” signal to ensure that autologous cells are not inappropriately phagocytosed.

While the absence of CD47 is known to result in phagocytosis, the effect of differential CD47 expression is unclear. *IAP*^{+/-} platelets and erythrocytes may be more prone to phagocytosis than their wild-type counterparts (Olsson et al., 2005, 2007), which suggests that CD47 downregulation might lead to clearance of these cells as they age. However, no formal exploration of the role of CD47 expression on normal or leukemic hematopoietic stem and progenitor cells, which are also physiologically migrating cells, has been undertaken. Hence, we performed experiments to understand the effect of differential CD47 expression on these cells.

RESULTS

CD47 Expression Is Increased in HSCs after Mobilization or Induced Inflammation

Mobilization of marrow stem and progenitor cells involves several steps in which they come into contact with macrophages. We

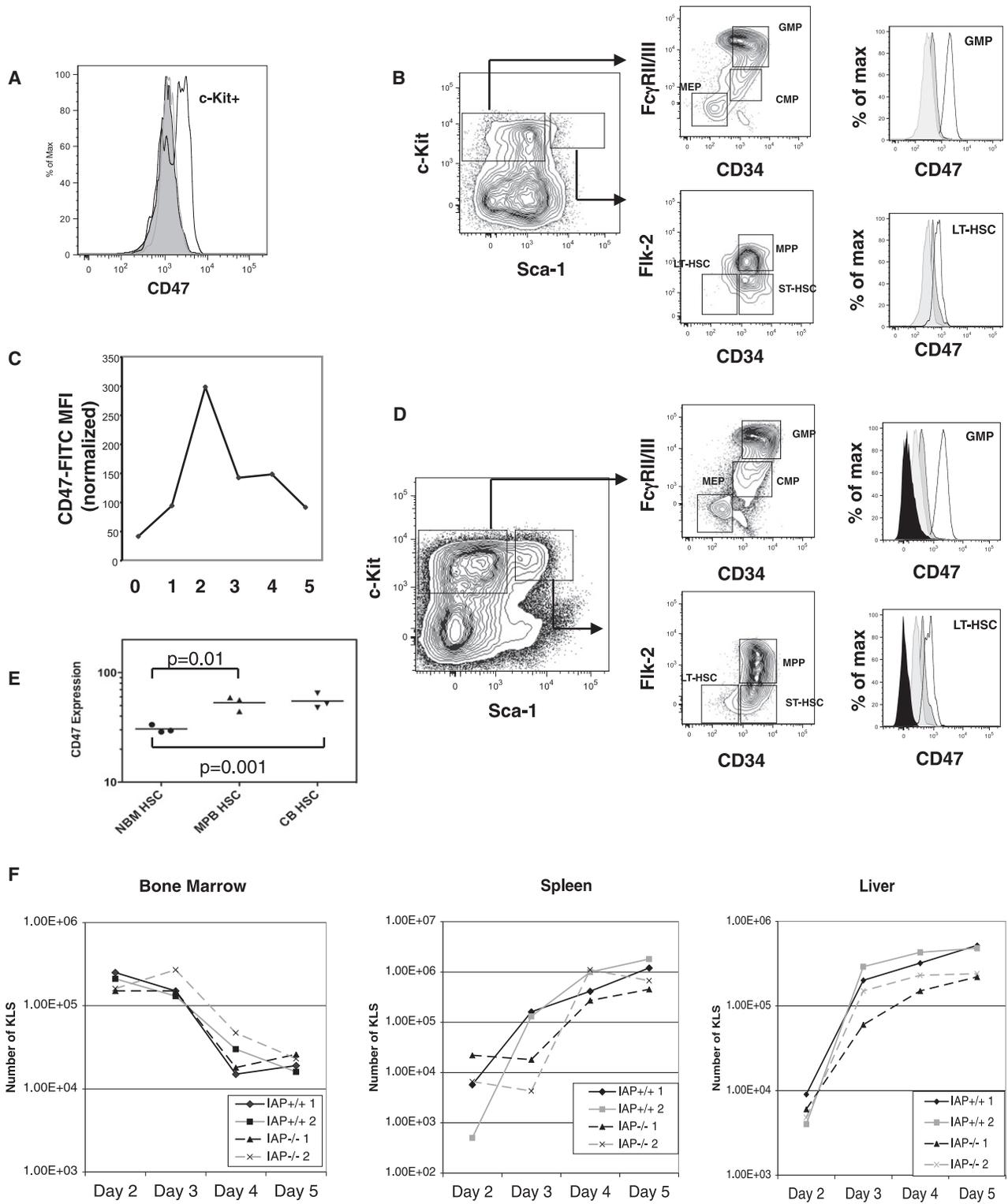


Figure 1. Mobilization or Inflammation Induces CD47 Upregulation in Hematopoietic Stem and Progenitor Cells

(A) Expression level of CD47 on cKit⁺ cells is shown for day 2 Cy/G-mobilized BM.

(B) Myeloid progenitor and stem cell gates are shown for day 2 mobilized BM. Histograms on right show level of CD47 expression in marrow LT-HSCs and GMPs for steady state (light gray shaded histogram), day 2 mobilized (black line), and day 5 mobilized (dark gray shaded histogram).

(C) Relative MFI of CD47 for GMPs on days 0–5 of Cy/G mobilization. Results were normalized so that steady-state GMPs had MFI 100.

thus utilized the cyclophosphamide/G-CSF (Cy/G) protocol (Morrison et al., 1997) to experimentally induce mobilization in mice. We found that there was a notable increase of CD47 on cKit⁺ bone marrow cells on day 2 (Figure 1A), when bone marrow HSCs reach their maximum level, with CD47 increasing approximately 4-fold by this time point (Figure 1B). The increase was seen at all levels of the myeloid progenitor hierarchy, as long-term hematopoietic stem cells (LT-HSCs) as well as granulocyte-macrophage progenitors (GMPs) displayed this increase in CD47 expression (Figure 1B). By day 5, when egress from the marrow to distant marrow/spleen/liver sites had nearly stopped, the levels of CD47 on the marrow HSC/progenitors had returned to nearly normal levels. In Figure 1C, the mean fluorescence intensity of CD47 expression on GMPs is shown on days 0 to 5 of mobilization.

These results predicted that mobilized human HSCs in the blood would also have increased levels of CD47 on their cell surfaces. We examined the CD47 expression level on human bone marrow, cord blood (CB), and mobilized peripheral blood (MPB) HSCs. The results show that human HSCs in circulation have significantly higher levels of CD47 expression than sessile HSCs (Figure 1E).

Endotoxins such as lipopolysaccharide (LPS) are also thought to contribute to bone marrow mobilization (Cline and Golde, 1977) by causing activation of macrophages and a proinflammatory response (Fenton and Golenbock, 1998), as well as increasing the phagocytic capacity of macrophages (Wright et al., 1989). We tested whether LPS administration in mice would affect CD47 expression in stem and progenitor cells. Mirroring the pattern seen in Cy/G-induced mobilization, LPS caused expansion of stem and progenitor cells by 2 days post-treatment, followed by migration to the spleen and liver (Figure 1D). On day 2 after LPS administration, stem and progenitor cells in the marrow had upregulated CD47 to a similar degree as in Cy/G mobilization. By day 5, when the inflammatory response had resolved, the levels of the protein had dropped to steady-state levels (Figure 1D).

CD47 Upregulation during Mobilization Is Unlikely to Be Necessary for Migration

Since CD47 was consistently upregulated in the mobilization response, we tested the ability of stem and progenitor cells to mobilize following Cy/G. The CD47 knockout mouse has defects in migration of neutrophils to sites of inflammation (Lindberg et al., 1996) and of dendritic cells to secondary lymphoid organs (Van et al., 2006). The exact role of CD47 in migration of these cells is unknown, but it may relate to poor integrin binding in the circulation or lack of interaction with SIRP α on endothelial cells. Hence we reasoned that if CD47 was involved in the migration capacity of these cells during mobilization, then *IAP*^{-/-} mice would display reduced numbers of cells in the peripheral organs after Cy/G.

We thus administered Cy/G to both wild-type and knockout mice and sacrificed mice on days 2–5. For each mouse, we

analyzed the number of stem and progenitor cells in peripheral blood, marrow, spleen, and liver. We used the KLS (lineage-negative, cKit-positive, Sca1-positive) population as a surrogate for HSCs because the number of CD34⁻ cells drops considerably in proliferative states, making accurate calculation of LT-HSC numbers difficult.

We found that there was little difference in numbers of mobilized KLS or GMPs between wild-type and *IAP*^{-/-} mice (Figure 1F). There was a modest decrease in the ability of *IAP*^{-/-} mice to move progenitors to the spleen by day 3 (about 10-fold less), but by days 4 and 5 they had restored normal numbers of cells to the periphery. The marrow and liver compartments in *IAP*^{-/-} had no difference in HSCs or GMPs from wild-type mice. Additionally, there was no difference in HSC numbers in peripheral blood by day 4 (data not shown). Hence, *IAP*^{-/-} mice do not have a significant mobilization defect. While this result does not support a role for CD47 in the migration phase of mobilization, it does not rule out a role for protection against phagocytosis since macrophages from *IAP*^{-/-} mice also fail to properly phagocytose CD47-deficient cells, likely due to aberrant macrophage education (Wang et al., 2007).

CD47-Deficient Mice Have No Gross Hematopoietic Defect, but *IAP*^{-/-} HSCs Fail to Engraft Wild-Type Mice

We examined stem and progenitor frequencies in *IAP*^{+/-} and *IAP*^{-/-} mice. The relative frequency of cells in the stem and myeloid progenitor compartment did not differ between these mice and wild-type mice (Figure 2A). We then tested stem cells from these mice for their ability to form colonies in an in vitro assay. We examined colony formation at day 7 and found that there was no major difference between wild-type and *IAP*^{-/-} stem cells in the number and type of colonies formed (Figure 2B).

We also tested whether bone marrow cells from *IAP*^{-/-} mice could rescue recipient mice from the effects of lethal irradiation. Typically, a dose of 2×10^5 bone marrow cells will rescue 100% of recipient mice in this assay. In agreement with previous results (Blazar et al., 2001), we found that *IAP*^{-/-} bone marrow could not rescue these recipients (Figure 2C). However, administration of these cells did prolong life span; normally, mice die between day 12 and 15 after irradiation, but mice that received *IAP*^{-/-} bone marrow lived about 7–10 days longer (Figure 2C). We do not yet know the reason for the prolongation of life span in this case.

Next, we sorted Fik2⁻ CD34⁻ KLS stem cells from wild-type and *IAP*^{-/-} cells and transplanted them into lethally irradiated wild-type recipients along with 2×10^5 competitor cells. None of the mice that received *IAP*^{-/-} HSCs had any engraftment of donor cells, indicating that CD47 was indeed required to be expressed intrinsically for the HSCs to transplant (Figures 2D and 2E). We speculated that this was due to phagocytosis of CD47 null cells, as has been shown for erythrocytes and T cells. To test this, we enriched cKit⁺ cells from the bone marrow of wild-type and *IAP*^{-/-} mice and coincubated them with bone marrow-derived macrophages. *IAP*^{-/-} stem and progenitor cells

(D) Myeloid progenitor and stem cell gates are shown for day 2 BM post-LPS treatment. Histograms show level of CD47 expression on day 2 post-LPS (black line), day 5 post-LPS (dark gray shaded histogram), steady state (light gray shaded histogram), and *IAP*^{-/-} (black shaded histogram).

(E) MFI for human CD47 on HSCs from human bone marrow (NBM), cord blood (CB), or mobilized peripheral blood (MPB).

(F) Evaluation of KLS cells in the hematopoietic organs of *IAP*^{+/-} and *IAP*^{-/-} mice mobilized on days 2 through 5. Two mice are analyzed per genotype per day.

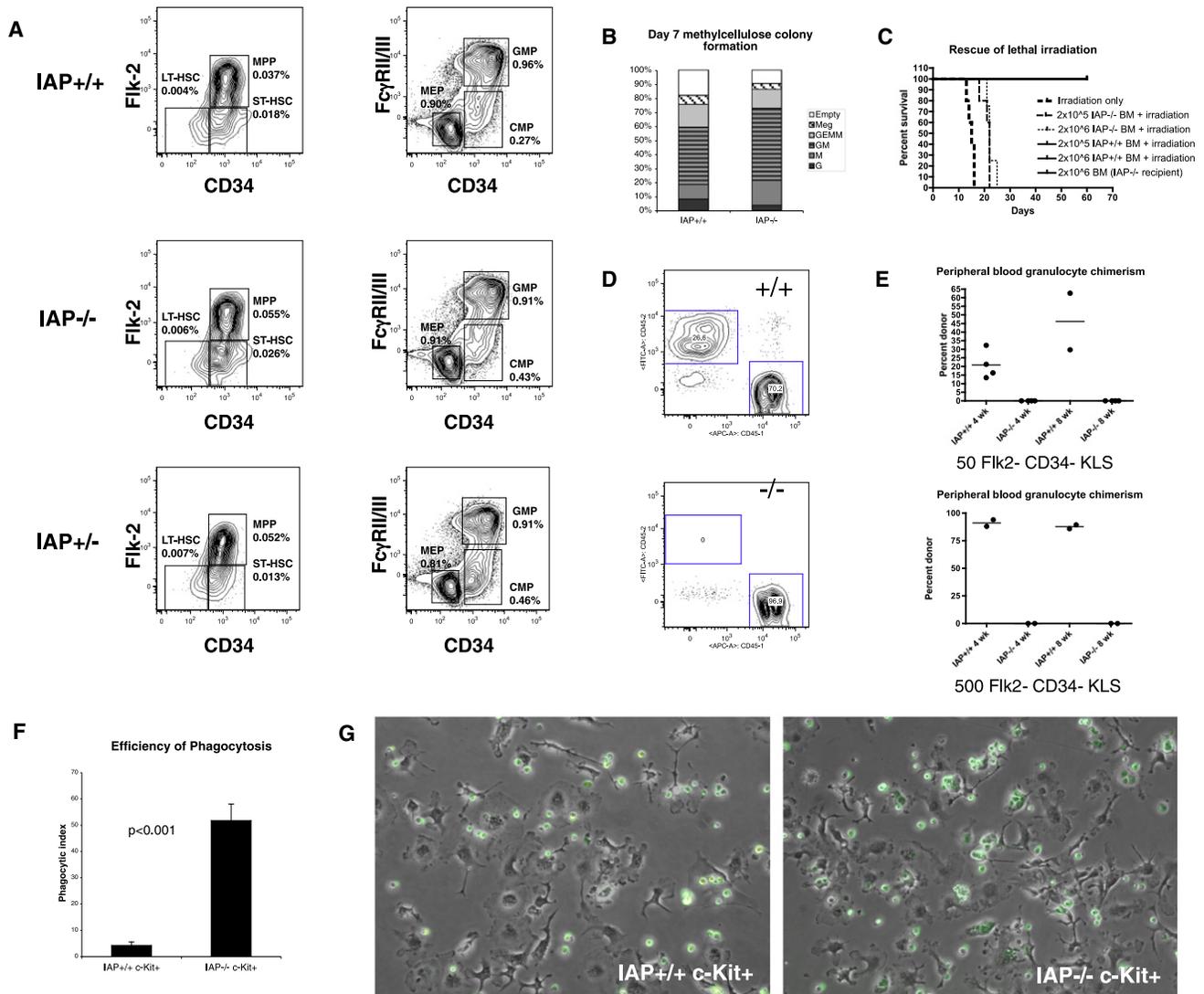


Figure 2. CD47-Deficient HSCs Are Efficiently Phagocytosed by Macrophages but Otherwise Exhibit Normal Hematopoietic Developmental Potential

(A) Stem cells (left column) are gated on Lin⁻ cKit⁺ Sca1⁺ cells. Myeloid progenitors (right column) are gated on Lin⁻ cKit⁺ Sca1⁺ cells. Frequency in whole bone marrow is shown adjacent to each gated population.

(B) Colony output on day 7 of individually sorted LT-HSCs. G: granulocyte; M: macrophage; GM: granulocyte and macrophage; GEMM: granulocyte, macrophage, erythroid, and megakaryocyte; Meg: megakaryocyte.

(C) Survival curve of recipient mice given lethal radiation and transplanted with the cells shown, n = 5 for each group.

(D) Examples of chimerism plots at 4 weeks post-transplant for IAP^{+/+} or IAP^{-/-} donors.

(E) Summary of chimerism analysis of mice transplanted with either 50 or 500 IAP^{+/+} or IAP^{-/-} cells.

(F) Results of phagocytosis assays using IAP^{+/+} or IAP^{-/-} cKit-enriched bone marrow. n = 3, error bars represent 1 standard deviation (SD).

(G) Photomicrographs of phagocytosis assays taken after 2 hr.

were readily phagocytosed in this assay, whereas wild-type cells were only minimally phagocytosed (Figures 2F and 2G). These results were not due to increased apoptosis of the IAP^{-/-} cells in these culture conditions, as there was no difference in Annexin V positivity between the groups (Figure S4A available online).

We also tested HSC migration using the parabiosis model in which two mice are joined surgically to allow their circulatory systems to form anastomoses and a shared blood system

(Wright et al., 2001b). This model allows the examination of migration in a more physiological setting because stem cells are continuously seeded into the blood stream from the marrow over time. We joined an IAP^{-/-} mouse with a congenic wild-type GFP⁺ mouse. For both pairs of mice that were parabiosed, bone marrow HSC chimerism was seen in the IAP^{-/-} mouse but not the wild-type mouse (Figure S1). Thus, IAP^{-/-} HSCs are cleared even during physiological migration.

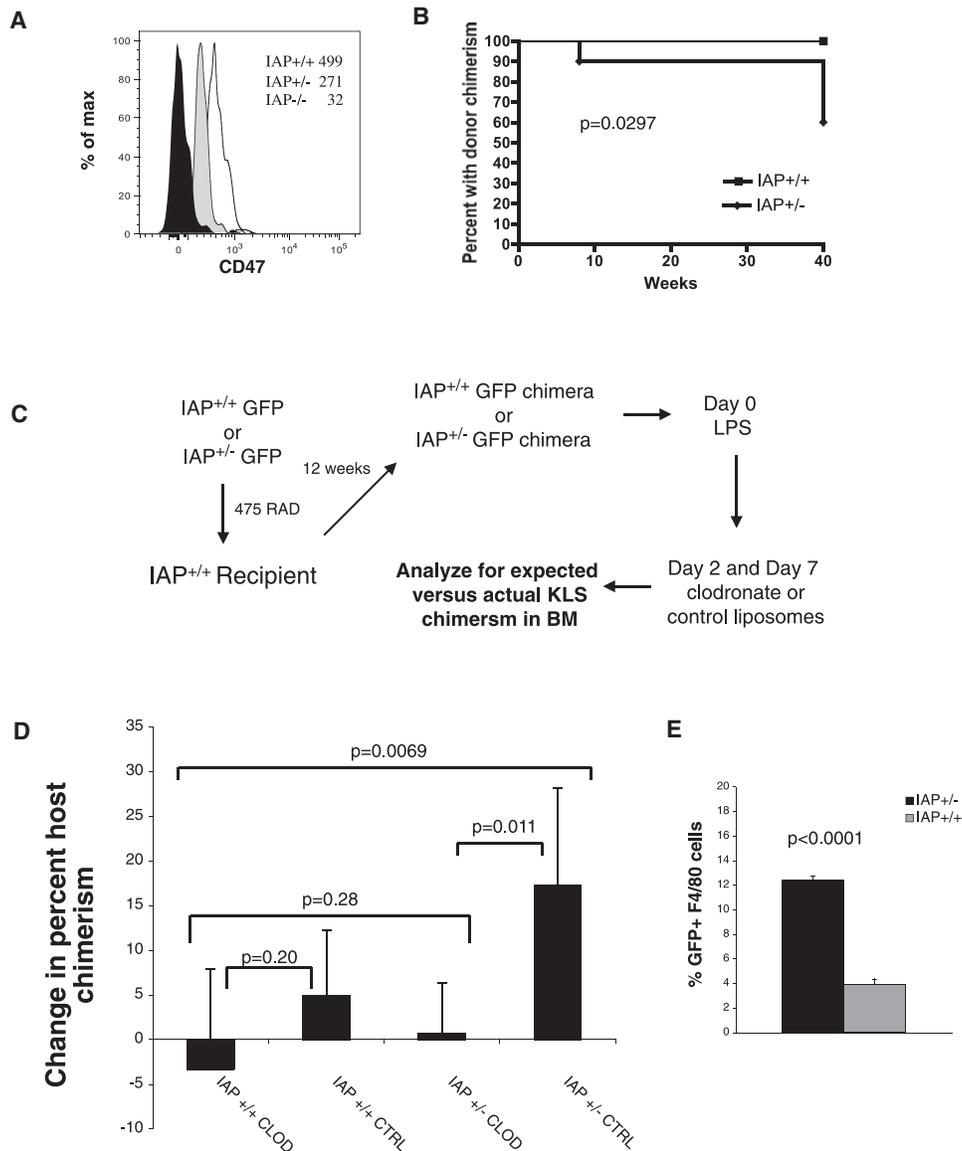


Figure 3. $IAP^{+/-}$ HSCs Have a Competitive Disadvantage Relative to Wild-Type HSCs due to Macrophages

(A) MFI of CD47 on $IAP^{+/+}$, $IAP^{+/-}$, and $IAP^{-/-}$ LT-HSCs.

(B) Donor chimerism analysis for transplants of $IAP^{+/+}$, GFP⁺ or $IAP^{+/-}$, GFP⁺ marrow cells. Loss of donor chimerism is shown using Kaplan-Meier curves.

(C) Experimental design for assessing effect of CD47 heterozygosity during LPS challenge.

(D) Change in percent chimerism of host KLS cells compared to expected chimerism based on peripheral blood granulocytes is shown. Error bars represent 1 SD and p values were obtained by ANOVA statistics. n = 5 for $IAP^{+/+}$ with clodronate (CLOD), n = 7 for $IAP^{+/+}$ with control (CTRL), n = 4 for $IAP^{+/-}$ with clodronate, n = 7 for $IAP^{+/-}$ with control.

(E) Results of in vivo phagocytosis assay comparing $IAP^{+/+}$ and $IAP^{+/-}$ cKit-enriched cells. Percent recipient GFP⁺ F4/80 cells is shown (n = 3 for each group, error bars represent 1 SD).

CD47 Heterozygous HSCs Have Reduced Fitness Relative to Wild-Type HSCs due to Macrophage Clearance

Our observation that CD47 expression increases in states of stress and mobilization led us to hypothesize that hematopoietic stem and progenitor cells (HSPCs) that were genetically hemizygous for CD47 might be more prone to phagocytosis and clearance by macrophages over time, as has been seen for platelets

and erythrocytes (Olsson et al., 2005, 2007). Hence, we asked if $IAP^{+/-}$ stem cells would be disadvantaged relative to wild-type stem cells in long-term contribution to hematopoiesis. We first analyzed the levels of CD47 expressed on $IAP^{+/+}$, $IAP^{+/-}$, and $IAP^{-/-}$ stem cells. FACS analysis of CD34⁻ Fik-2⁻ KLS stem cells revealed that the mean fluorescence intensity (MFI) of CD47 on heterozygote HSCs was indeed at roughly half the level of that on wild-type stem cells (Figure 3A).

We then transplanted one cohort of sublethally irradiated recipients with 2×10^6 wild-type whole bone marrow cells and another with the same dose of $IAP^{+/-}$ bone marrow cells. Such a dose would be expected to contain roughly 50–100 HSCs. Since granulocyte chimerism in the peripheral blood is a good surrogate marker of stem cell fitness (Bhattacharya et al., 2006), we analyzed cells from the blood of these recipients at periodic intervals. When wild-type marrow was transplanted into wild-type recipients, granulocyte chimerism was maintained for at least 40 weeks. However, when $IAP^{+/-}$ cells were transplanted, 4 out of 10 mice lost donor chimerism over time, despite having a successful engraftment initially ($p = 0.0297$, Figures 3B and S2A). It was possible that there were fewer LT-HSCs per unit of marrow in $IAP^{+/-}$ or $IAP^{-/-}$ mice compared to wild-type. To test if $IAP^{-/-}$ mice had fewer functional HSCs, we transplanted either $IAP^{+/+}$ or $IAP^{-/-}$ marrow cells into $IAP^{-/-}$ donors. We also cotransplanted $IAP^{+/-}$ marrow into wild-type mice together with wild-type marrow. We did not find any statistical difference in donor granulocyte chimerism for up to 12 weeks in either case (Figures S2B and S2C), indicating that there was not a demonstrable deficit in stem cell numbers in $IAP^{+/-}$ or $IAP^{-/-}$ mice.

Interestingly, the $IAP^{+/-}$ chimeras that did not lose donor engraftment over time did not appear to have a quantitative deficit in chimerism; rather they seemed to have levels of donor cells similar to those in the wild-type chimeras (Figure S2A). We thus hypothesized that physiological insults leading to chronic or acute inflammatory states that occurred in some mice, but not others, led to their losing $IAP^{+/-}$ donor chimerism over time, but at steady state the donor $IAP^{+/-}$ cells were not affected.

To test this, we created chimeras by transplanting whole bone marrow from either $IAP^{+/+}$ GFP⁺ or $IAP^{+/-}$ GFP⁺ mice into sublethally irradiated wild-type syngeneic recipients. Since $IAP^{-/-}$ mice have no known in vivo (Lindberg et al., 1996) or in vitro (Figure 2B) defect in granulopoiesis, blood granulocyte chimerism can be used in this setting to estimate donor HSC engraftment. After 12 weeks, contribution of donor HSCs was assessed. At this early time point, $IAP^{+/-}$ mice actually had a higher blood granulocyte contribution, but there was considerable variation within each group. The mice were challenged with a sublethal dose of LPS (day 0), then given clodronate or control liposomes on day 2 and day 7. Clodronate liposomes have been shown to specifically but transiently deplete marrow, spleen, and liver macrophages when injected intravenously (Van Rooijen and Sanders, 1994). At day 14, the mice were euthanized and actual marrow KLS chimerism was assessed and compared to expected chimerism based on blood granulocyte chimerism (Figure 3D). Raw values are shown in Table S1. The results indicate that there were more host (wild-type) KLS cells in the bone marrow of the $IAP^{+/-}$ chimeras than expected. When macrophages were depleted, the chimerism was at the level that would be expected based on blood granulocyte chimerism. ANOVA showed a significant interaction among IAP genotype ($IAP^{+/+}$ versus $IAP^{+/-}$) and treatment with and without clodronate (CLOD versus CTRL) ($p = 0.0069$). When the group including the $IAP^{+/-}$ chimeras treated with control liposomes ($IAP^{+/-}$ CTRL) was excluded, there was no statistical difference between the groups ($p = 0.28$). Analysis also showed that differences between the treatment with and without clodronate were not

significant in $IAP^{+/+}$ mice ($p = 0.20$) but were significant in $IAP^{+/-}$ mice ($p = 0.011$).

It was possible that these results were due to increased apoptosis of $IAP^{+/-}$ cells relative to $IAP^{+/+}$. To test this, we measured the percent of Annexin V⁺ 7-AAD⁻ KLS cells in $IAP^{+/+}$ and $IAP^{+/-}$ mice treated with LPS in the presence of control or clodronate liposomes. There was no difference between the groups (Figure S4B).

To establish that macrophages were indeed responsible for clearing the cells with reduced CD47 expression, we challenged donor $IAP^{+/+}$, GFP⁺ and $IAP^{+/-}$, GFP⁺ mice with LPS. After 3 days, we harvested bone marrow from these mice and transplanted cKit-enriched cells intrasplenically into GFP-negative wild-type recipient mice similarly challenged with LPS 3 days prior. After 2 hr the recipient spleens were harvested and analyzed by FACS. There were significantly more F4/80⁺ macrophages that were GFP⁺ (representing macrophages containing phagocytosed GFP⁺ cells) in mice injected with $IAP^{+/-}$ cells compared to $IAP^{+/+}$ ($p < 0.0001$, Figures 3E and S3).

CD47 Is Upregulated on Mouse Myeloid Leukemias

Previous microarray analysis had revealed that CD47 was significantly upregulated in leukemic *Fas^{lpr/lpr} × hMRP8bcl2* transgenic bone marrow (Traver et al., 1998), and this was confirmed in leukemic *hMRP8bcr/abl × hMRP8bcl2* mice (Jaiswal et al., 2003). Transcripts for CD47 were found by quantitative RT-PCR to be increased in leukemic *hMRP8bcr/abl × hMRP8bcl2* bone marrow 3- to 4-fold and 6- to 7-fold in cKit-enriched leukemic marrow relative to healthy *hMRP8bcl2*⁺ bone marrow (Figure 4E). Leukemic mice had an expansion of the GMP population as well as cKit⁺ Sca1⁺ Lin⁻ stem and progenitor subsets in the spleen relative to control mice, which were of the same genotype as leukemic mice but failed to develop disease (Figures 4A–4D). Expression levels for CD47 protein were found to begin increasing in leukemic mice relative to control mice at the stage of the Flk2⁻ CD34⁻ cKit⁺ Sca1⁺ Lin⁻ LT-HSC (Figure 4F) (Yang et al., 2005). This increased level of expression was maintained in GMP and Mac-1⁺ blasts but not megakaryocyte/erythroid restricted progenitors (MEP) (Figure 4F). The increase in CD47 surface expression between leukemic and normal cells was between 3- and 20-fold, depending on the sample and fluorophore used (Figure S5C). We have observed increased CD47 expression in all mice that developed leukemia from *hMRP8bcr/abl × hMRP8bcl2* primary ($n = 3$) and secondary transplanted mice ($n = 3$), *Fas^{lpr/lpr} × hMRP8bcl2* (Figure S4A) primary ($n = 14$) and secondary ($n = 19$) mice, and *hMRP8bcl2 × hMRP8bcl2* primary ($n = 3$) and secondary ($n = 12$) mice. We have also found increased CD47 expression in mice that received p210bcr/abl retrovirally transduced mouse bone marrow cells and then developed leukemia (Figure S5B).

CD47 Expression on Normal and Leukemic Human Stem Cells

Because we found CD47 to be increased on all mouse leukemias we examined, we investigated CD47 expression on normal and dysplastic human hematopoietic cells. FACS-mediated analysis of human hematopoietic progenitor populations was performed on blood- and marrow-derived cells from normal CB and MPB

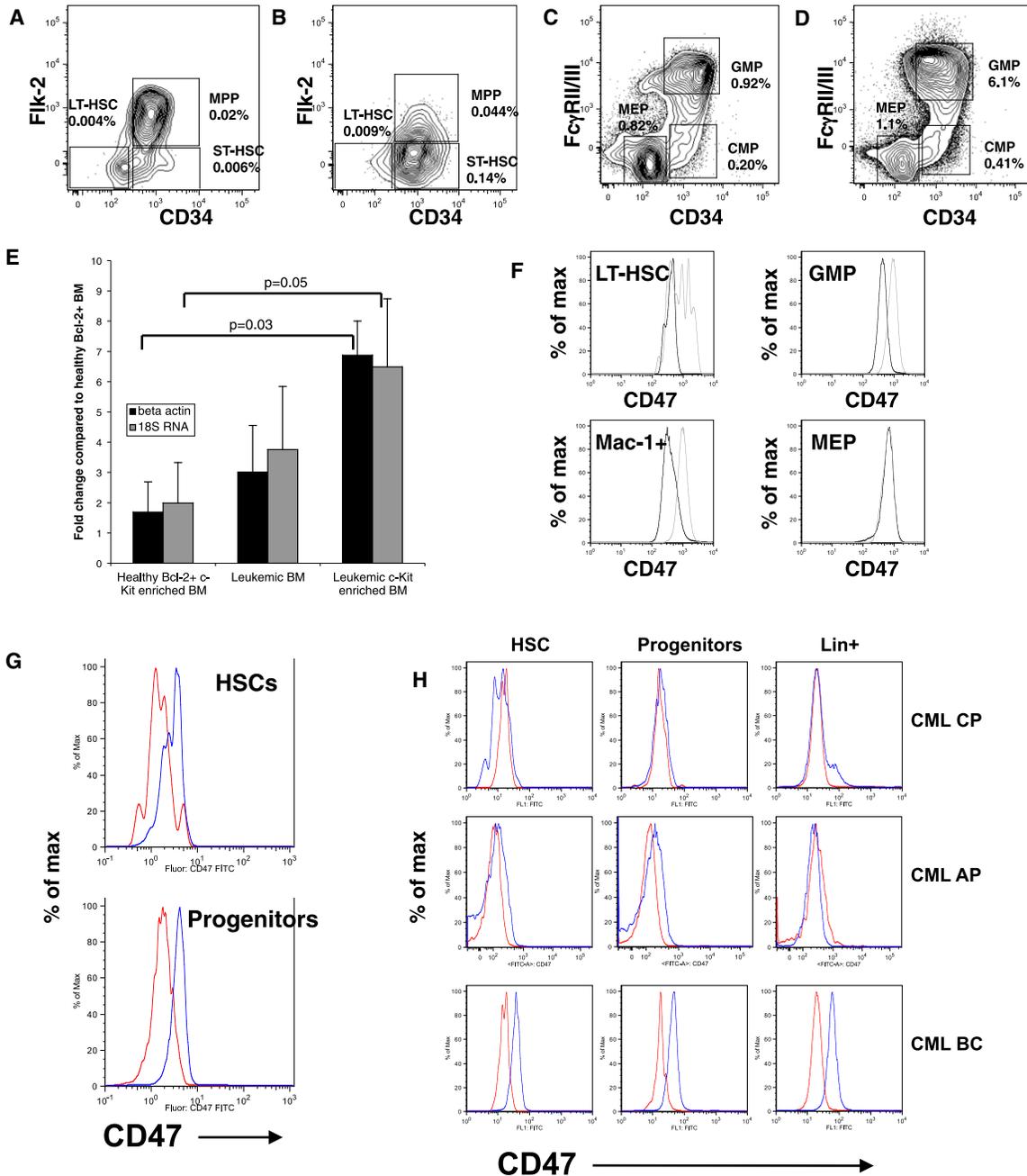


Figure 4. CD47 Is Upregulated in Murine and Human Myeloid Leukemia

Typical stem and progenitor plots are shown for leukemic *hMRP8bcrabl × hMRP8bcl2* cells compared to control nonleukemic animals. Lin⁻ cKit⁺ Sca1⁺ gated cells from control bone marrow (A) and leukemic spleen (B) and Lin⁻ cKit⁺ Sca1⁻ gated cells from control bone marrow (C) and leukemic spleen (D). Frequency is shown as a percentage of entire marrow or spleen mononuclear fraction.

(E) Quantitative RT-PCR for CD47. Data are shown from three sets of mice transplanted with either leukemic or control *hMRP8bcrabl × hMRP8bcl2* BM cells and then sacrificed 2–6 weeks later. Results were normalized to beta-actin and 18S rRNA expression. Fold change relative to control transplanted whole Bcl-2⁺ BM cells was determined. Error bars represent 1 SD.

(F) Histograms show expression of CD47 on gated populations for leukemic (gray) and control (black) mice.

(G) Comparative FACS histograms of human CD47 expression by normal (red; n = 6) and acute myeloid leukemic (AML, blue; n = 6) hematopoietic stem cells (HSCs; CD34⁺CD38⁻CD90⁺Lin⁻) and progenitors (CD34⁺CD38⁺Lin⁻).

(H) Comparative FACS histograms of CD47 expression by normal (red) and chronic myeloid leukemia (blue) hematopoietic stem cells (HSCs; CD34⁺CD38⁻CD90⁺Lin⁻), committed progenitors (CD34⁺CD38⁺Lin⁻), and Lin⁺ cells.

(n = 16) and myeloproliferative disorders (MPDs) including polycythemia vera (PV; n = 16), myelofibrosis (MF; n = 5), essential thrombocythemia (ET; n = 7), chronic myelomonocytic leukemia (CMML; n = 11), and atypical chronic myeloid leukemia (aCML; n = 1), as well as myeloid blast crisis (BC) phase chronic myeloid leukemia (CML; n = 19), chronic phase CML (n = 7), and acute myeloid leukemia (AML; n = 13). This analysis demonstrated that the GMP population was expanded in MPDs with myeloid skewed differentiation potential including atypical CML, proliferative phase CMML, and acute leukemia including blast crisis CML and AML (Figures S6A and S6C). AML HSCs and progenitors uniformly exhibited higher levels of CD47 expression compared with normal controls (Figure 4G); every sample from CML-BC (n = 19) and AML (n = 13) had elevated levels of CD47. Moreover, progression from chronic phase CML to blast crisis was associated with a significant increase in CD47 expression (Figure 4H). Using the methods described in this study, we have found that human CD47 protein expression in CML-BC increased 2.2-fold in CD90⁺ CD34⁺ CD38⁻ Lin⁻ cells relative to normal CB and MPB ($p = 6.3 \times 10^{-5}$), 2.3-fold in CD90⁻ CD34⁺ CD38⁻ Lin⁻ cells relative to normal ($p = 4.3 \times 10^{-5}$), and 2.4-fold in CD34⁺ CD38⁺ Lin⁻ cells ($p = 7.6 \times 10^{-6}$) (Figures 4G, 4H, and S6B). Since CB and MPB HSCs have almost double the amount of CD47 as normal marrow (Figure 1E), these figures likely underestimate the true increase in CD47 compared to normal marrow.

We also examined CD47 expression in other myeloproliferative disorders, such as polycythemia vera, post-polycythemic myeloid metaplasia with myelofibrosis, essential thrombocythemia, agnogenic myeloid metaplasia, and chronic myelomonocytic leukemia. None of the samples we examined from these disorders displayed increased CD47 (data not shown). It is likely that the fixed, high expression of CD47 on AML cells and CML-BC cells represents a late event in malignant progression, whether by genetic or epigenetic heritably increased expression. A summary of all the human samples analyzed is shown in Table S2.

CD47 Expression Can Rescue the In Vivo Growth Defect of the AML Cell Line MOLM-13 via a Mechanism Other Than Migration

In order to experimentally test the significance of elevated CD47 expression on myeloid leukemias, we decided to examine whether the level of CD47 expressed on myeloid leukemia could affect its pathogenicity in vivo. We first screened a panel of human AML cell lines for human CD47 expression. On the basis of this analysis, we found significant variation in the amount of cell surface CD47 (adjusted for cell size) in the varying lines (Figures S7A and S7B). We then tested whether there was variation in the ability of some of these cell lines to engraft in immunocompromised mice. Human HL-60 and Kasumi-1 cells, which express moderate to high levels of human CD47, were able to engraft C57Bl/6 or Balb/c recombination-activating gene 2, common gamma chain-deficient (RAG2^{-/-}, $\gamma c^{-/-}$) mice (Figures S6C and S6D), which are reported to have little or no interaction between their SIRP α and human CD47 (Takenaka et al., 2007). MOLM-13 cells, which are derived from a patient with AML 5a, have low endogenous CD47 expression and were unable to

engraft in either of these strains. Even when transplanted into nonobese diabetic severe combined immunodeficiency, interleukin 2 receptor common gamma chain^{-/-} (NOG) mice that are reported to have more effective interaction between their mouse SIRP α and human CD47, and thus should be permissive for human donor cells (Takenaka et al., 2007), MOLM-13 cells failed to engraft (Figure S7C).

Since MOLM-13 cells failed to engraft in any of the mouse models tested, we decided to use them for further analysis of the role of CD47 in leukemogenicity. MOLM-13 cells were transduced with either control (Tet) or CD47-expressing (Tet-CD47) lentiviruses (Figure 5A), and stable integrants were propagated on the basis of GFP expression. We first tested if ectopic expression of the murine CD47 (muCD47) protein could rescue the growth defect of these cells. The cells were transplanted intravenously in a competitive setting with untransduced MOLM-13 cells into T-, B-, and NK-deficient RAG2^{-/-}, $\gamma c^{-/-}$ mice. Only cells transduced with Tet-CD47 were able to give rise to tumors in these mice, efficiently engrafting bone marrow, spleen, liver, and peripheral blood (Figures 5B and 5C).

Since CD47 has been reported to be important for the migration of hematopoietic cells, one possibility for the lack of growth of Tet MOLM-13 cells in mice was their inability to migrate to niches. To test this, Tet MOLM-13 or Tet-CD47 MOLM-13 cells were directly injected into a single femoral cavity of immunodeficient Balb/c mice. Tet-CD47 MOLM-13 cells were able to spread to all bones and other hematopoietic tissues of recipient mice and lead to death, whereas Tet MOLM-13 cells had minimal, if any, engraftment and only at the site of injection, without morbidity by 75 days after transplant (Figures 5D–5F). These results suggested a function other than or in addition to migration for CD47 in MOLM-13 engraftment.

The Level of CD47 Expressed on MOLM-13 Cells Determines Tumorigenic Potential

Our results up to this point had indicated that ectopically expressing muCD47 on a nonengrafting human cell line could rescue its growth defect. These experiments did not, however, answer whether the level of CD47 expressed on a leukemic cell could affect its tumor-forming potential. To model the tumorigenic effect of having high versus low CD47 expression, we sorted clones of muCD47 expressing MOLM-13 cells into high and low expressors. When adjusted for cell size, CD47 density on the CD47^{lo} MOLM-13 cells was approximately equal to mouse bone marrow cells, whereas CD47^{hi} MOLM-13 cells had 9-fold higher expression, an increase commensurate with the change seen in CD47 expression on primary leukemic cells compared to their normal counterparts (Figures 6A and 6B). When high- or low-expressing cells were transplanted into recipients, only mice transplanted with high-expressing cells succumbed to disease by 75 days of age (Figure 6C). Furthermore, organomegaly was more pronounced in these mice (Figure 6D). As expected, the infiltration of MOLM-13 cells in bone marrow and spleen of recipient mice was also much higher for mice transplanted with CD47^{hi} MOLM-13 cells as well (Figure 6E). We also examined the level of CD47 expression in two mice that received CD47^{lo} MOLM-13 cells but had significant marrow engraftment. In both cases, the persisting cells after 75 days had

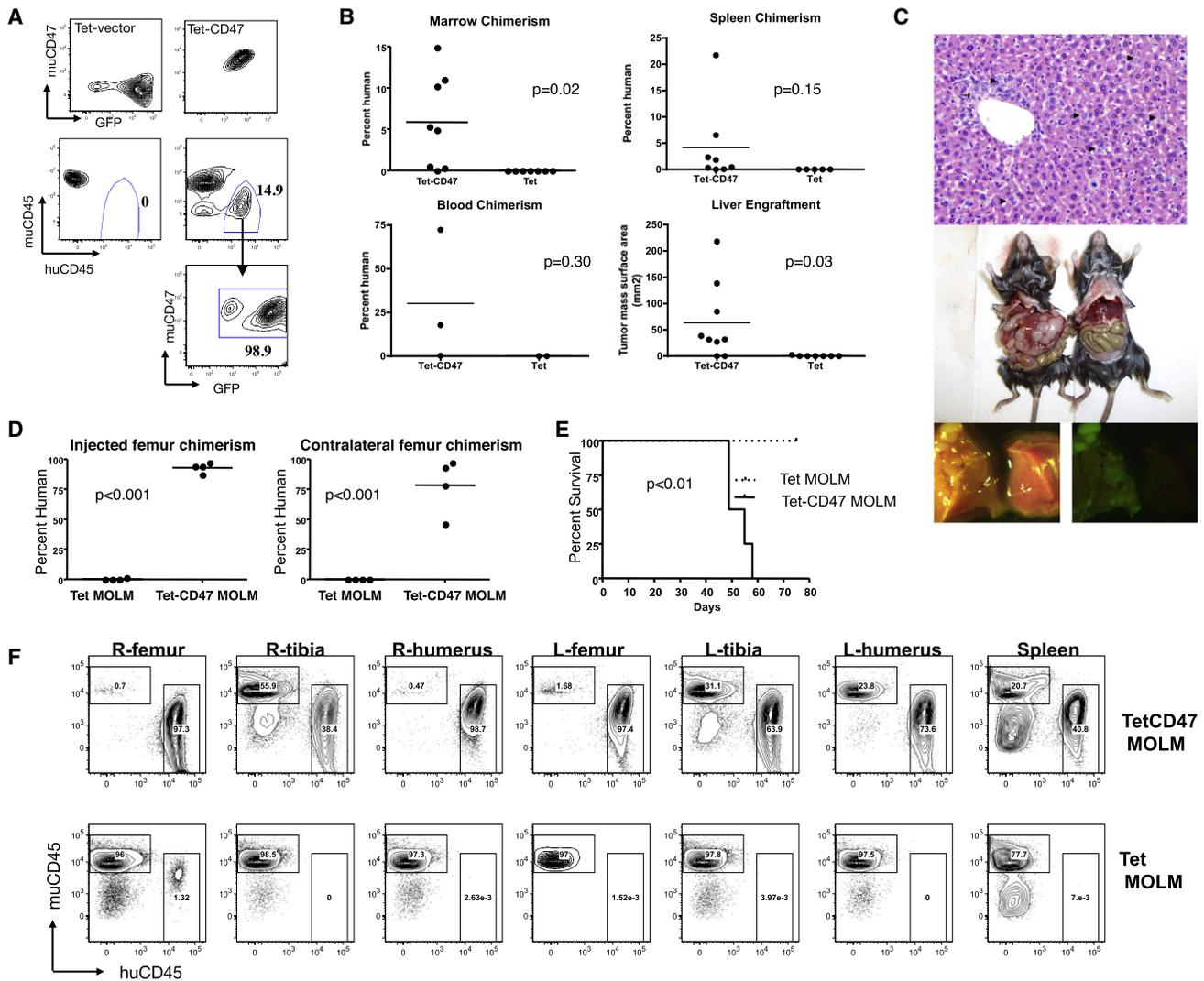


Figure 5. Overexpression of Murine CD47 Rescues the Growth Defect of MOLM-13 Myeloid Leukemia Cells

(A) GFP and human CD45 chimerism for mice transplanted with untransduced MOLM-13 cells (5×10^5) and either 5×10^5 Tet ($n = 6$) or Tet-CD47 MOLM-13 ($n = 8$) cells.

(B) MOLM-13 chimerism in hematopoietic tissues was determined by human CD45 chimerism and measurement of tumor lesion size.

(C) Hematoxylin and eosin sections of Tet-CD47 MOLM-13 transplanted liver (200 \times) (top panel). Periportal (arrow) and sinusoidal (arrowhead) tumor infiltration is evident. Examples of liver tumor formation and hepatomegaly in Tet-CD47 MOLM-13 transplanted mice versus control transplanted mice (middle panel). GFP fluorescence demonstrates tumor nodule formation as well diffuse infiltration (bottom panel).

(D) 1×10^6 Tet ($n = 5$) or Tet-CD47 MOLM-13 ($n = 4$) cells were injected into the right femur of RAG2^{-/-}, Gc^{-/-} mice and the tissues were analyzed 50–75 days later for chimerism of MOLM-13 cells in BM.

(E) Survival curve of mice transplanted intrafemorally with Tet or Tet-CD47 MOLM-13 cells.

(F) Representative FACS plots from mice transplanted intrafemorally with Tet or Tet-CD47 MOLM-13 cells. R, right; L, left.

much higher levels of CD47 than the original line (Figure 6F), indicating that a strong selection pressure exists in vivo for high levels of CD47 expression on MOLM-13 leukemia cells.

We were also able to modulate CD47 expression with doxycycline because we utilized the Tet-responsive (Tet-OFF) promoter element to control expression of CD47 in MOLM-13 cells. Beginning 2 weeks after transplantation with CD47^{hi} MOLM-13 cells, a cohort of mice was given doxycycline to extinguish CD47 expression and followed for up to 75 days post-transplant.

During this time, none of the mice given doxycycline succumbed to disease or had large infiltration of MOLM-13 cells in hematopoietic organs (Figures 6D and 6E) other than 1–2 large extraparenchymal masses seen in the livers. At the dose of doxycycline used in this experiment, muCD47 expression in MOLM-13 cells was reduced to levels below that of normal mouse bone marrow but notably was not completely absent (Figure 6B). Thus, a sustained high level of CD47 expression is required for robust MOLM-13 growth in hematopoietic organs.

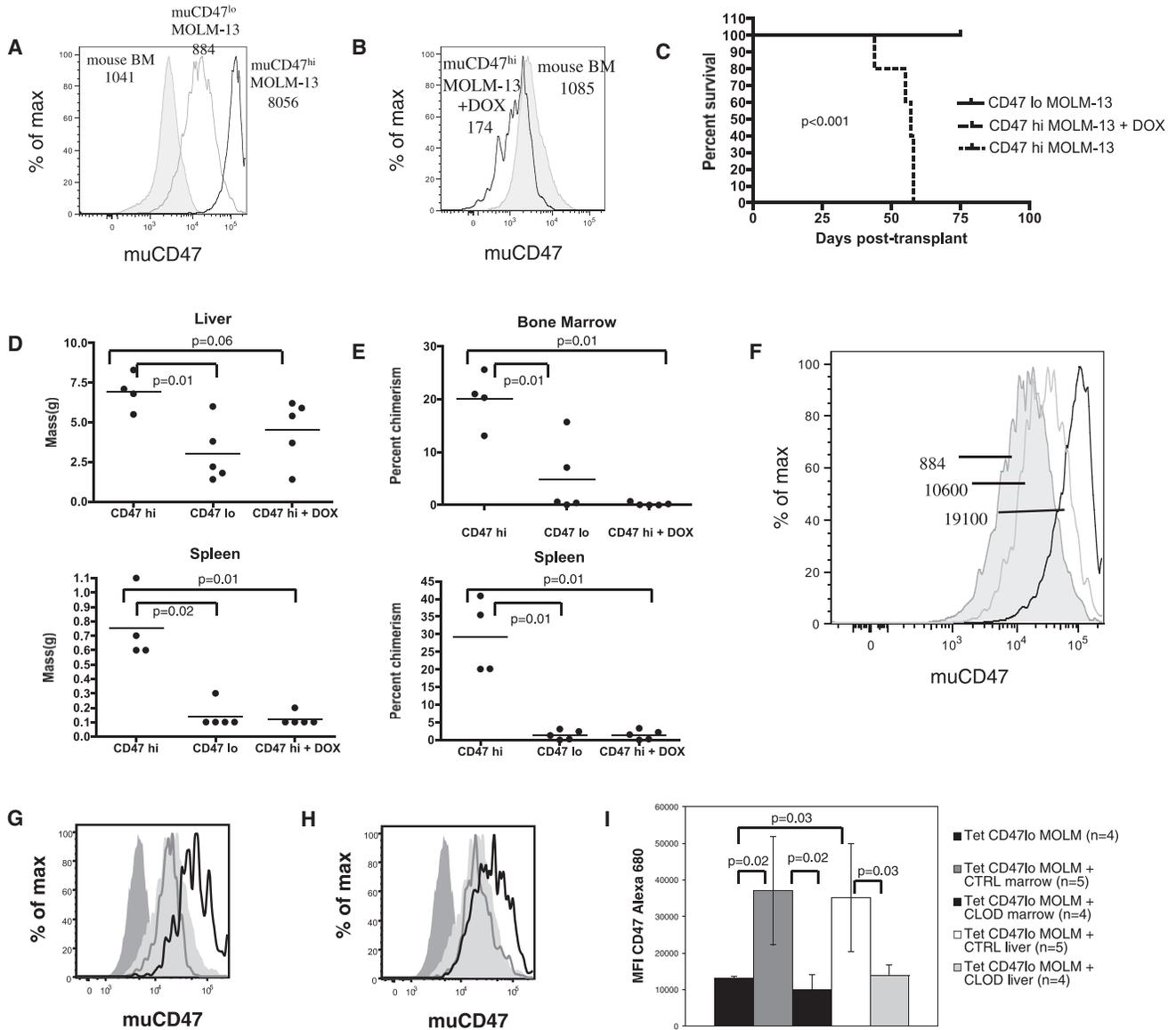


Figure 6. Higher Expression of CD47 on MOLM-13 Cells Causes Increased Tumorigenicity

(A) Histograms show CD47 expression in MOLM-13 high (black), MOLM-13 low (gray), and mouse bone marrow (shaded) cells, with MFI normalized for size shown.

(B) The histograms show level of CD47 expression on CD47^{hi} MOLM-13 cells in untreated (shaded) and doxycycline-treated (shaded) mice, with MFI normalized for size shown.

(C) Survival of mice transplanted with 1×10^6 CD47^{hi} MOLM-13 cells, CD47^{lo} MOLM-13 cells, or CD47^{hi} MOLM-13 cells with doxycycline administration started after 2 weeks post-transplant. $n = 5$ for CD47^{hi} and CD47^{hi} plus doxycycline. $n = 10$ for CD47^{lo}.

(D) Liver and spleen size of mice at necropsy or 75 days after transplant with 1×10^6 CD47^{hi} MOLM-13 cells, CD47^{lo} MOLM-13 cells, or CD47^{hi} MOLM-13 cells with doxycycline.

(E) Bone marrow and spleen chimerism of human cells in mice at necropsy or 75 days after transplant with 1×10^6 CD47^{hi} MOLM-13 cells, CD47^{lo} MOLM-13 cells, or CD47^{hi} MOLM-13 cells with doxycycline.

(F) Murine CD47 expression on CD47^{lo} MOLM-13 cells engrafting in bone marrow (open) compared with original cell line (shaded), with MFI normalized for size shown.

(G) Bone marrow and (H) liver histograms are shown for MOLM-13 cells in mice 35 days after transplant with 1×10^6 CD47^{lo} MOLM-13 cells. Parental Tet MOLM-13 (dark gray shaded) and Tet CD47^{lo} MOLM-13 CD47 (light gray shaded) expression, as well as engrafting cells in the clodronate (gray line) and control (black line) cohorts, is displayed in the histograms.

(I) MFI of CD47 channel in engrafting MOLM-13 cells in clodronate (CLOD) or control (CTRL) treated mice is shown for bone marrow and liver. Error bars represent 1 SD.

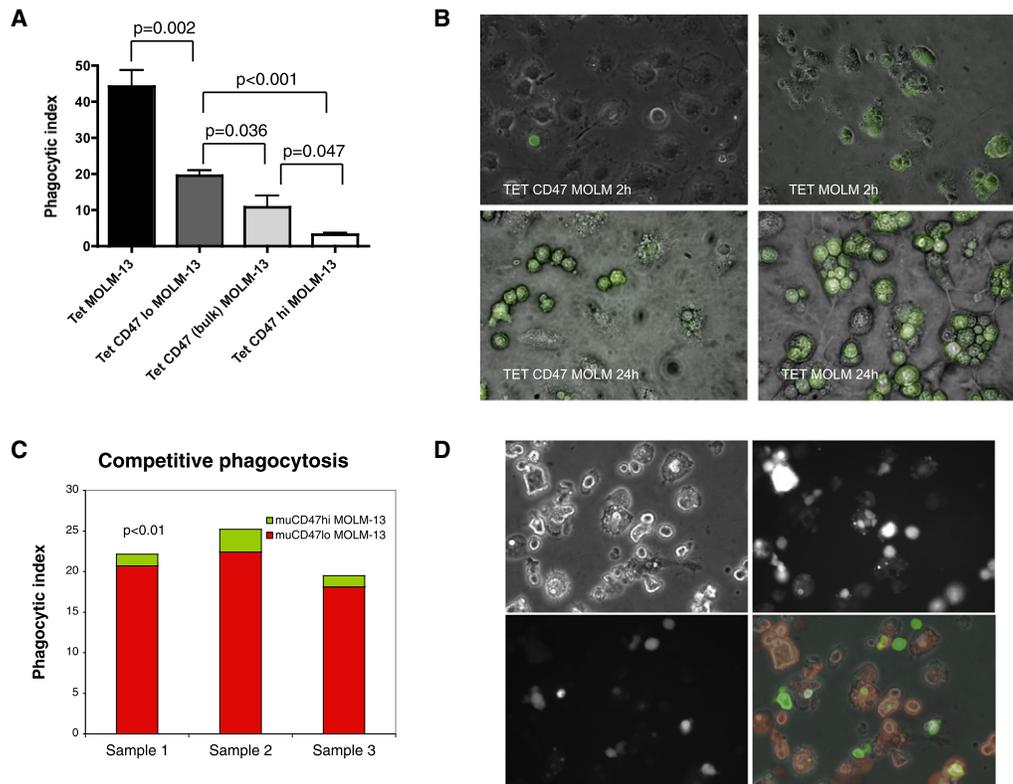


Figure 7. Increasing CD47 Expression on MOLM-13 Cells Confers Increasing Ability to Evade Macrophage Phagocytosis

(A) Tet, Tet-CD47^{lo}, Tet-CD47 bulk, or Tet-CD47^{hi} MOLM-13 cells were incubated with bone marrow-derived macrophages (BMDM) for 2 hr and phagocytic index was determined. Error bars represent 1 SD (n = 6 for each time point).

(B) Photomicrographs of BMDMs incubated with Tet or Tet-CD47 MOLM-13 cells at 2 and 24 hr (400x).

(C) CD47^{hi} GFP and CD47^{lo} MOLM-13 RFP cells were coincubated with BMDMs for 2 hr. Phagocytic index is shown for three separate samples for CD47^{hi} GFP (green) and CD47^{lo} MOLM-13 RFP (red) cells.

(D) Photomicrographs show brightfield (top left), RFP (top right), GFP (bottom left), and merged (bottom right) images of CD47^{hi} GFP and CD47^{lo} MOLM-13 RFP cells that were coincubated with BMDMs for 24 hr.

Macrophages Mediate Selective Pressure for CD47^{hi} Variants of MOLM-13 Cells

Because we had observed that mice transplanted with CD47^{lo} MOLM-13 cells had selection over time for higher-expressing clones *in vivo*, we wondered if this selection pressure was mediated by macrophages. To test this, we used clodronate liposomes to deplete macrophages prior to and after transplant of CD47^{lo} MOLM-13 cells and sacrificed mice after 5 weeks. When control liposomes were used, the level of CD47 increased significantly on the engrafted human cells. However, when clodronate-containing liposomes were used, the expression of CD47 on MOLM-13 cells was maintained at the level of the parental line (Figures 6G–6I). Thus, macrophages mediate selective pressure for high-expressing CD47 clones *in vivo*.

Interestingly, when clodronate was used to deplete macrophages in the immunodeficient mice, the level of engraftment was comparable between the control Tet MOLM-13 cells and the CD47 MOLM-13 cells (Figure S8), suggesting that depletion of macrophages could compensate for lack of CD47 expression on MOLM-13 cells as well.

Ability to Evade Macrophage Phagocytosis Correlates with CD47 Expression Level

We incubated Tet (vector only), bulk (unsorted) Tet-CD47, Tet-CD47^{hi}, or Tet-CD47^{lo} MOLM-13 cells with bone marrow-derived macrophages (BMDM) for 2–24 hr and assessed phagocytosis by counting the number of ingested GFP⁺ cells under a microscope (Figures 7A and 7B) or by evaluating the frequency of GFP⁺ macrophages by flow cytometry (Figure S9A). There was a strong correlation between level of CD47 expressed and the likelihood of phagocytosis (Figure 7A). When CD47^{lo} RFP and CD47^{hi} GFP MOLM-13 cells were coincubated with macrophages in the same wells, the low-expressing cells were far more likely to be phagocytosed (Figures 7C and 7D). Thus, in a mixed population of cells with varying levels of CD47 expression, the low expressors were more likely to be cleared by macrophages over time. These results were not due to differences in apoptosis between CD47-expressing and control MOLM-13 cells, as both groups had similar levels of Annexin V positivity *in vitro* (Figure S4C).

We also injected MOLM-13 cells into mice and analyzed hematopoietic organs 2 hr later for evidence of macrophage

phagocytosis. Macrophages in bone marrow, spleen, and liver all had a higher GFP⁺ fraction when injected with Tet MOLM-13 cells as compared to muCD47-expressing cells, indicating that the muCD47⁻ cells were more likely to be phagocytosed *in vivo* as well (Figure S9B).

Finally, we asked if inhibiting macrophage SIRP α using a blocking antibody could abrogate the protection against phagocytosis that CD47 provided MOLM-13 cells. Indeed, when this receptor was blocked, CD47-expressing MOLM-13 cells were phagocytosed at the same rate as control MOLM-13 cells (Figure S10).

DISCUSSION

Physiological Significance of CD47 Upregulation

We demonstrate here that HSPCs upregulate CD47 in response to an insult that induces mobilization. This then leads to the surprising finding that HSPCs themselves are regulated by phagocytosis *in vivo*. Hence, our results indicate that protection from phagocytosis is essential for HSC survival during migration to the periphery after a strong mobilizing or inflammatory stimulus. A recent report indicates that HSPCs can circulate through the lymphatic system as well (Massberg et al., 2007). It will be interesting to see if these HSPCs upregulate CD47 to evade the numerous sinusoidal macrophages in lymph nodes.

Since leukemias upregulate CD47 as well, it appears that they co-opt this normal physiological response to stress-induced mobilization as a means to protect themselves at the expense of normal, non-neoplastic progenitors. In this manner, leukemic stem cells could quickly gain a survival advantage over normal stem cells, leading to their eventual take-over of the hematopoietic environment.

The regulatory mechanisms for CD47 expression in mobilization and leukemia remain unknown. The fact that CD47 is upregulated and downregulated in such a temporally precise manner during mobilization suggests that there is tight control over its expression in normal physiology. Leukemias, however, seem to have constitutively high CD47 expression. The only published regulator of CD47 expression is the transcription factor nuclear respiratory factor 1 (Nrf1) (Chang and Huang, 2004). It remains to be determined if Nrf1 or other regulatory elements are involved in the progression of human myeloid leukemia, and if there is a common regulatory pathway that operates in mobilization as well.

Macrophages Are Important Mediators of Tumor Immunosurveillance

Many examples of tumor clearance by T, B, and NK cells have been described in the literature, indicating that a healthy immune system is essential for regulating nascent tumor growth. However, to date, few examples indicating that macrophage-mediated phagocytosis can check tumor development have been produced. Collectively, our studies reveal that ectopic overexpression of CD47 can enable otherwise immunogenic tumor cells to grow rapidly in a T, B, and NK cell-deficient host. Furthermore, this is likely to reflect a mechanism used by primary human myeloid leukemias to evade the host immune system since CD47 is upregulated in nearly all sufficiently

advanced murine and human myeloid leukemias examined thus far.

This form of immune evasion is particularly important since these cancers occupy sites of high macrophage infiltration. The leukemias studied here are clonal yet found throughout the marrow (and in mouse, spleen, and liver); these are organs with abundant intra-tissue macrophages as well as macrophage-lined sinusoids at the entry site of circulating blood cells. CD47 was first cloned as an ovarian tumor cell marker, indicating that it may play a role in preventing phagocytosis of other tissue cancers as well (Campbell et al., 1992). Furthermore, solid tumors often metastasize to macrophage-rich tissues such as liver, lung, bone, and lymph nodes, indicating that they must be able to escape macrophage-mediated killing in those tissues. Preventing CD47-SIRP α interaction could be doubly effective because antigens from phagocytosed tumor cells may be presented by professional antigen-presenting cells such as macrophages or dendritic cells to activate an adaptive immune response, leading to further tumor destruction.

CD47 May Be Upregulated because Some Cancers Are More Immunogenic Than Normal Tissue

But why would leukemic progenitor cells require additional protection against phagocytosis? Like aged neutrophils or erythrocytes, tumor cells can display signs of cellular “damage” and/or glycosidic variations on their cell surface. There are well-established data that tumors can be recognized by NK cells via ligands that mark “stressed self” (Gasser et al., 2005). We propose that leukemic cells likewise express stress ligands that mark them for phagocytosis; by upregulating CD47, this innate immunity check on tumor growth can be circumvented. One of these pro-phagocytic ligands, calreticulin, is known to be upregulated in aged cells. It has been shown that its effects can be counteracted by expression of CD47 (Gardai et al., 2005). It remains to be seen if this or other stress ligands are expressed on nascent leukemic cells to mark them for destruction.

Therapeutics Targeting CD47 May Be Clinically Useful for Treating Cancer

Others have shown that antibodies that crosslink CD47 on the surface of chronic lymphocytic leukemia cells can induce a caspase-independent cell death (Mateo et al., 1999; Uno et al., 2007). This phenomenon, while promising, appears to function primarily by inducing apoptosis, not necessarily by promoting phagocytosis. In fact, we show in a companion paper (Majeti et al., 2009 [this issue of *Cell*]), that anti-CD47 blocked CD47^{hi} human AML cells are phagocytosed by macrophages, without first becoming apoptotic.

In addition, our recent results in human AML indicate that increasing CD47 transcript levels are associated with shortened survival (Majeti et al., 2009), allowing the level of expression of this gene to potentially be used as an independent prognostic variable. Combined with the data presented here, where we show that CD47 expression levels affect the fitness of both normal HSCs and leukemic cells, we propose that CD47 is a compelling target for therapeutic strategies that reduce its functional interaction with macrophage SIRP α and thus render these cancers susceptible to innate and adaptive immune system clearance.

EXPERIMENTAL PROCEDURES

Mice

CD47-deficient *IAP*^{-/-} mice on a C57Bl/6 background were obtained from Eric Brown and bred onto our wild-type colony. *IAP*^{-/-} mice were crossed to beta-actin GFP (Wright et al., 2001a) or CD45.1 C57Bl/6 mice from our colony to obtain GFP⁺ or congenic strains. *hMRP8bcrabl*, *hMRP8bcl2*, and *Fas*^{lpr/lpr} transgenic mice were obtained as previously described (Traver et al., 1998; Jaiswal et al., 2003). C57Bl/6 mice from our colony were used as a source of wild-type cells. C57Bl/6 or Balb/c RAG2^{-/-} common gamma chain (Gc)^{-/-} mice were bred in our laboratory. NOD.Cg-Prkdcscidll2rgtm1Wjl/SzJ (NOG) mice were obtained from The Jackson Laboratory and bred in a pathogen-free environment at our facility. For transplant experiments, cells were transplanted into either immunodeficient mice given a radiation dose of 4 Gy using gamma rays from a cesium irradiator (Phillips), or CD45.2 C57Bl/6Ka mice given a radiation dose of 9.5 Gy. Sublethal radiation was given at a dose of 4.75 Gy. Mice were euthanized when moribund.

Cells were injected via tail vein in sterile PBS using a 27-gauge needle. Donor and recipient mice were 6–12 weeks of age. Parabiosis was performed as previously described (Wright et al., 2001b). Mice were mobilized with cyclophosphamide (Sigma) (200 mg/kg) and G-CSF (Neupogen) (250 µg/kg) as previously described (Morrison et al., 1997). Bacterial LPS from *E. coli* 055:B5 (Sigma) was administered at a dose of 40 mg/kg into the peritoneal cavity.

Cell Lines

MOLM-13 cells were obtained from DSMZ. HL-60, Jurkat, U937, K562, and Kasumi-1 cells were obtained from ATCC. Cells were maintained in Iscove's modified Dulbecco's media (IMDM) plus 10% fetal bovine serum (FBS) (Hyclone).

Quantitative RT-PCR Analysis

Bone marrow was obtained from leukemic *hMRP8bcr/abl* × *hMRP8bcl2* mice or *hMRP8bcl2* control mice. Cells were cKit enriched using cKit microbeads and an autoMACS column (Miltenyi) and cDNA was created from RNA using standard methods (Invitrogen, SuperScript II). cDNA corresponding to approximately 1000 cells was used per PCR reaction. Quantitative PCR was performed with a SYBR green kit on an ABI Prism 7000 PCR (Applied Biosystems) machine.

Cell Staining and Flow Cytometry

Mouse tissues were prepared and stained for stem, progenitor, and peripheral blood cells as previously described (Akashi et al., 2000; Yang et al., 2005; Bhattacharya et al., 2006). Mouse CD47 antibody (clone mIAP301, ATCC) was assessed using biotinylated antibody produced in our lab followed by staining with streptavidin-conjugated Quantum Dot 605 (Chemicon). Samples were analyzed using a FACSAria (Beckton Dickinson).

Normal human bone marrow and blood samples were obtained with informed consent from 20- to 25-year-old paid donors who were hepatitis A, B, C, and HIV negative by serology (All Cells). Leukemic blood and marrow cells were obtained with informed consent from previously untreated patients at Stanford University Medical Center according to IRB approved methods. HSCs and progenitors were stained and identified as previously described (Manz et al., 2002). Anti-human CD47 FITC (clone B6H12, PharMingen) was used to assess CD47 expression in all human samples. Following staining, cells were analyzed using a modified FACS Vantage (Becton Dickinson) or a FACSAria.

Engraftment of MOLM-13 cells was assessed by using anti-human CD45 PE-Cy7 (PharMingen), anti-mouse CD45.2 APC (clone AL1-4A2 produced in our lab), and anti-mouse CD47 Alexa-680 (mIAP301, produced in our lab).

All samples were resuspended in propidium iodide containing buffer before analysis to exclude dead cells. FACS data was analyzed using FloJo software (Treestar).

Lentiviral Preparation and Transduction

pRRL.sin-18.PPT.Tet07.IRES.GFP.pre, CMV, VSV, and tet trans-activator (TA) plasmids were obtained from Luigi Naldini (Vigna et al., 2002). The full-

length murine cDNA for CD47 form 2 was provided by Eric Brown (Genentech). Plasmid DNA was transfected into 293T cells and supernatant harvested and concentrated by standard protocols. Cells were transduced with lentivirus for 48 hr and GFP⁺ cells were sorted to purity and grown for several generations to ensure stability of the transgenes.

In Vitro Phagocytosis Assays

Bone marrow-derived macrophages were prepared and harvested by incubation in trypsin/EDTA (GIBCO) for 5 min and gentle scraping. Macrophages were plated at 5 × 10⁴ cells per well in a 24-well tissue culture plate (Falcon) for 24 hr. Fresh media were added 2 hr before 2.5 × 10⁵ target cells were added to the macrophage-containing wells and incubated at 37°C for the indicated times. After coincubation, wells were washed thoroughly with IMDM three times and examined under an Eclipse T5100 (Nikon) using an enhanced green fluorescent protein (GFP) or Texas Red filter set (Nikon). Phagocytic index was calculated using the following formula: phagocytic index = number of ingested cells/(number of macrophages/100). At least 200 macrophages were counted per well.

Macrophage Depletion

Clodronate was a gift of Roche Diagnostics GmbH. It was encapsulated in liposomes as previously described (Van Rooijen and Sanders, 1994). Macrophages were depleted by injecting intravenously (i.v.) 200 µl of the final liposome preparation 48 hr prior to cell transplant. Mice were injected with 100 µl every week thereafter to maintain depletion until they were euthanized.

Statistics

Statistics were calculated using Prism 4.0 software (GraphPad Software, Inc.) The Welch corrected student's t test was used to calculate all column statistics. Two-tailed p values are shown in figures. For survival curves, p values were obtained using a logrank test. For comparison of groups, one-way ANOVA and tests of contrast were used where indicated.

Additional Experimental Procedures are available in Supplemental Data.

SUPPLEMENTAL DATA

Supplemental Data include two tables, ten figures, and Supplemental Experimental Procedures and can be found with this article online at [http://www.cell.com/supplemental/S0092-8674\(09\)00651-5](http://www.cell.com/supplemental/S0092-8674(09)00651-5).

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Author contributions: S.J., C.H.M.J., W.W.P., M.P.C., R.M., and I.L.W. designed experiments. S.J., C.H.M.J., W.W.P., C.Y.P., M.P.C., and D.T. performed experiments and analyzed data. N.v.R. provided reagents. S.J., C.H.M.J., and I.L.W. wrote the manuscript. All authors endorse the full content of this work.

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